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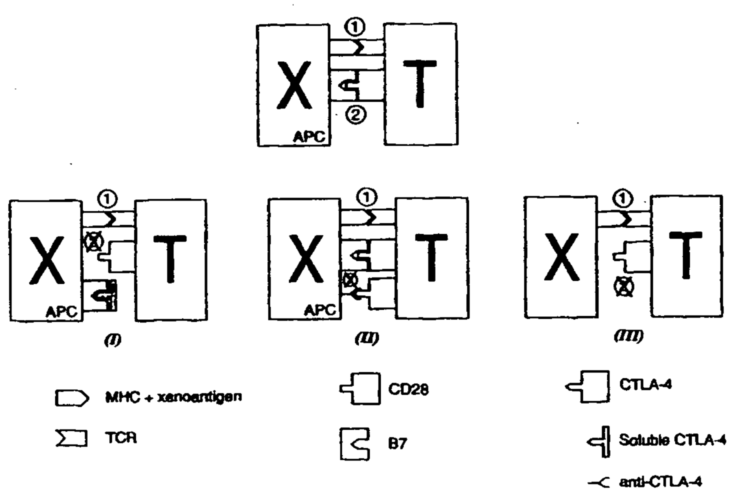

(57) Abstract

The invention provides means and methods for inhibiting T-cell mediated rejection of a xenotransplanted organ by blocking the delivery of co-stimulatory signal 2 (the B7/CD28 interaction) in order to prevent the activation of xenoreactive T-cells in the recipient. In a first aspect, co-stimulation is prevented by administration to the organ recipient of a soluble form of CTLA-4 from the xenogeneic donor organism. This preferentially binds B7 on the xenograft and blocks the interaction between B7 on the xenogeneic donor cells and CD28 on recipient T-cells. In a second aspect, co-stimulation is antagonised by expressing a ligand for CTLA-4 on the xenogeneic donor cells. This ligand binds to CTLA-4 on activated T-cells of the recipient and antagonises signal 2. In a third aspect, co-stimulation is prevented by expressing recipient organism MHC class II on the surface of the cells of the xenogeneic donor organ. The donor cells are thus able to present xenoantigens to a recipient T-cell in the context of self-MHC class II. If the donor cells do not express B7, or if B7 is blocked, the xenoreactive recipient T-cell becomes anergic.

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It appears that the suppression of anti-xenograft indirect T-cell responses will be one of the greatest challenges for xenotransplantation [5,6]. Maintaining the level of immunosuppression required to prevent chronic xenograft rejection due to persistent indirect immunogenicity may be unfeasible using conventional systemic immunosuppressive drugs because of the increased the risks of infection and neoplasia [*eg.* 7]. Clearly, if xenotransplantation is to be clinically successful, methods to promote graft-specific immunosuppression are needed in order to reduce the requirements for systemic therapy.

T-cell activation requires two separate signals. Delivery of signal 1 alone induces a refractory state ("anergy"), defined as the inability to produce IL-2 after subsequent antigenic exposure. For full activation to occur, the cell must be co-stimulated with signal 2.

In vivo, signal 1 is provided by the interaction of the TCR/CD4 complex with either allogeneic MHC or antigenic peptide complexed with self MHC; signal 2 is supplied by the interaction between B7 molecules (B7.1 and B7.2, also known as CD80 and CD86, respectively) on the antigen-presenting cell (APC) and CD28 on the T-cell

Monoclonal antibodies (mAbs) have played a key role in studying T-cell activation. Signal 1 can be supplied by mAbs directed against the TCR/CD3 complex, and mAbs against CD28 can provide signal 2. Indeed, T-cells can be activated by two suitable mAbs, even in the absence of APC. Activation can also be prevented, rather than provided, using mAbs. Signal 2 can be blocked, for instance, using mAbs which block either B7 or CD28.

Signal 2 can also be blocked by using modified forms of CTLA-4, a high-affinity ligand for B7. CTLA-4 is a natural negative regulator of T-cell activation, and B7 binding to CTLA-4 on an activated T-cell antagonises the co-stimulatory signal provided by the B7/CD28 interaction. Soluble forms of CTLA-4, consisting of the extracellular domains of CTLA-4 linked to the constant domain of an antibody, have been constructed [8,9] to block T-cell activation. These molecules ("CTLA4-Ig" or "CTLA4-Fc") behave in a similar way to anti-B7 antibodies and have been used *in vitro* and *in vivo* to prevent the co-stimulatory functions of B7 and thus promote tolerance [10].

Targeting the B7/CD28 interaction to prevent T cell sensitisation to graft antigens *in vivo* has been shown to be an effective strategy to enhance graft survival. Using CTLA4-Ig, prolonged survival has been obtained in various allograft models [*eg.* 11] and in a human-to-murine islet xenograft model [12]. In the xenograft model, CTLA4-Ig administration caused full tolerance against the xenoantigens by rendering direct-reactive T cells anergic.

IMMUNOSUPPRESSION BY BLOCKING T CELL CO-STIMULATION SIGNAL 2 (B7/CD28 INTERACTION)

This invention relates to the suppression of xenograft rejection.

BACKGROUND TO THE INVENTION

The success of allogeneic organ transplantation has been established in the last few decades, but the limited supply of donor organs means that many patients have little or no chance of receiving a transplanted organ, such as a kidney, heart or liver. A significant number of these people die whilst awaiting an organ. One potential solution is "xenografting", or the use of organs from a non-human ("xenogeneic") animal donor.

Porcine donor organs are thought to be suitable candidates because pigs are anatomically and physiologically similar to humans and are in abundant supply. Porcine organs are rejected rapidly upon revascularisation, however, by a humoral process called hyperacute rejection (HAR). This is caused by naturally-occurring antibodies in the recipient which recognise and cross-react with antigens on the endothelial cells (ECs) of the xenograft. This recognition triggers the complement cascade which in turn leads to rejection.

European patent 0495852 (Imutran) suggests that membrane-bound regulators of host complement should be expressed on the xenograft in order to prevent the complete activation of complement in the organ recipient. This approach has been developed and applied in order to produce transgenic animals with organs designed to survive hyperacute rejection [*eg.* refs 1 & 2].

However, organs surviving HAR are subject to delayed xenograft rejection (DXR). This is characterised by the infiltration of recipient inflammatory cells and thrombosis of graft vessels, leading to ischaemia. WO98/42850 shows that expression of coagulation inhibitors on the surface of the xenograft can inhibit the thrombotic aspect of this type of rejection.

HAR and DXR are followed by the host T lymphocyte-mediated response. There are two pathways, "direct" and "indirect" by which T-cells may become sensitised against xenoantigens. The direct pathway involves interactions between T-cells and MHC molecules on xenogeneic donor cells, whereas the indirect pathway involves the presentation of processed xenoantigens by host APCs in the context of MHC class II. The indirect T-cell response is much stronger against xenoantigens than against alloantigens [3], which contrasts with findings for the direct pathway [4], indicating that both the direct and indirect human T-cell responses against xenoantigens must be suppressed if xenotransplantation is to be effective.

The invention thus provides a method of inducing xenotransplant tolerance in an organ recipient, comprising the administration to said recipient of a soluble form of the CTLA-4 protein from the xenogeneic donor organism.

The soluble form of CTLA-4 preferably comprises a fragment of the CTLA-4 from the donor organism which retains the ability to bind B7. This fragment is preferably the complete extracellular domain of CTLA-4.

Preferably, the soluble protein further comprises the constant domain of an immunoglobulin (*eg.* the Cγ1 chain of IgG1). Preferably, this is from the recipient organism, in order to prevent an immune response against this portion of the molecule.

In a typical embodiment for pig-to-human transplantation, therefore, the soluble protein could comprise the extracellular domain of porcine CTLA-4 fused to a human Cγ1 sequence.

Soluble forms of CTLA-4 from other organisms are described in, for instance, references 8 (human CTLA-4/human Ig γ1 constant region) and 9 (murine CTLA-4/human Ig γ1).

The invention also provides the use of a soluble form of xenogeneic CTLA-4 in the preparation of a medicament for inducing xenograft tolerance in an organ recipient.

The protein may be administered before, during, or after the xenotransplantation. Pre-xenotransplantation administration is most useful when the recipient is undergoing a pre-transplantation immunisation programme involving exposure to xenogeneic cells.

In the context of a pig being the donor organism, the invention provides a protein comprising the amino acid sequence shown in Figure 2 as SEQ ID:1, which is CTLA-4 cloned from porcine cells. This is the preferred form of CTLA-4 for use in the invention. The extracellular domain of this protein is also shown in Figure 2.

The invention also provides nucleic acid which encodes protein SEQ ID:1 (or fragments thereof). This preferably comprises the nucleotide sequence shown in Figure 3 as SEQ ID:2.

In addition, the invention provides a vector comprising the nucleic acid of the invention, and a cell transformed with such a vector.

The second aspect

In a second aspect, co-stimulation by signal 2 is antagonised by expressing a ligand for CTLA-4 on the xenogeneic donor cells. This ligand binds to CTLA-4 on activated T-cells of

It is thus an object of the invention to provide means to promote xenograft-specific immunosuppression. In particular, it as an object of the invention to inhibit T-cell-mediated rejection of xenotransplanted organs by preventing the organ recipient's T-cells from mounting an immune response against the organ. More specifically, it is an object to prevent this immune response by inducing anergy in the recipient's T-cells which recognise the xenotransplanted organ, resulting in xenograft-specific T-cell tolerance.

DESCRIPTION OF THE INVENTION

The invention provides methods and biological reagents for inhibiting T-cell mediated rejection of a xenotransplanted organ by blocking the delivery of co-stimulatory signal 2 in order to prevent the activation of xenoreactive T-cells in the recipient.

This is embodied in three aspects, which are illustrated in Figure 1. It will be appreciated that these three aspects can be used in isolation or in various combinations. Furthermore, conventional immunosuppressive techniques may be used alongside those of the invention.

The following should be read in conjunction with the section entitled "Definitions", which begins on page 8.

The first aspect

In a first aspect, co-stimulation by signal 2 is prevented by administration to the organ recipient of a soluble form of CTLA-4 from the xenogeneic donor organism. If, for instance, a pig organ (donor) were being transplanted into a human (recipient), a soluble form of porcine CTLA-4 (see below) would be administered to the human.

Although CTLA-4 from one organism (*eg.* pig) is able to bind to B7 from another organism (*eg.* human), the highest avidity is found for allogeneic B7. Whilst soluble CTLA-4 from the donor organism can thus bind to both recipient B7 (on normal cells) and donor B7 (on xenotransplanted cells), it preferentially binds B7 on the xenograft. This results in xenograft-specific immunosuppression, unlike the administration of CTLA-4 from the recipient organism, which would tend to lead to systemic immunosuppression. By blocking the interaction between B7 on the xenogeneic donor cells and CD28 on recipient T-cells, co-stimulatory signal 2 is not delivered to the T-cell of the recipient. Xenoreactive recipient T-cells are therefore rendered anergic.

So that the cell can engage recipient T-cells, the cell preferably also expresses MHC (class I or class II) on its surface. Suitably, therefore, the cell of the invention is a donor professional APC. Because of the antagonistic signal provided by the anti-CTLA-4 protein, however, these professional APC behave functionally as B7-negative cells.

The invention also provides biological tissue comprising such a cell.

The invention further provides an animal comprising a cell and/or biological tissue according to the second aspect.

The invention also provides a process for rendering biological tissue suitable for xenotransplantation, comprising the step of treating said biological tissue such that it expresses one or more proteins according to the second aspect on the surface of its cells.

The invention also provides a method of transplantation comprising the step of transplanting biological tissue according to the invention from a donor animal (*eg.* a pig) into a xenogeneic recipient animal (*eg.* a human).

In addition, the cells of the invention are suitable for pre-transplantation administration. This results in tolerance being induced in recipient T-cells before the xenograft itself is transplanted. Whilst the cells used in such pre-transplantation regimes should preferably express MHC class II, it will be appreciated that the cells need not be professional APCs.

Furthermore, the invention provides protein or nucleic acid according to the second aspect for use as a medicament.

The invention also provides the use of protein, nucleic acid, a vector, or a delivery system according to the second aspect in the manufacture of a formulation for administration to a xenotransplant recipient or donor.

The third aspect

In a third aspect, co-stimulation by signal 2 is prevented by expressing recipient organism MHC class II on the surface of the cells of the xenogeneic donor organ. If, for instance, a pig organ (donor) were being transplanted into a human (recipient), the pig organ would express human MHC class II.

Even if direct activation of recipient T-cells is avoided, for instance by utilising one or both of the first two aspects of the invention described above, indirect activation can still occur, involving the processing and presentation of xenoantigens on MHC class II by recipient APC.

the recipient and antagonises the co-stimulatory signal provided by the interaction between donor B7 and recipient CD28. This renders xenoreactive T-cells anergic.

The invention thus provides a membrane-associated protein which can bind to CTLA-4.

This will typically be a chimeric protein (*ie.* a protein produced by combining regions of different proteins into a single protein) comprising a CTLA-4-binding region and a membrane-association region. In its simplest form, the protein will thus be a fusion protein

By "membrane-associated protein", it is meant that the protein is attached to a cell membrane such that its extracellular domain can bind to CTLA-4. In order to attach the protein to the cell membrane, the protein might comprise a transmembrane sequence from a membrane protein, for instance, or a GPI anchor. A preferred transmembrane sequence is that of CD4 or CD8. Alternatively the protein might include a sequence which enables it to associate extracellularly with a membrane protein without the protein itself being inserted into the cell membrane.

It may also be desirable for the protein to comprise the cytoplasmic domain which is usually associated with said transmembrane regions (*eg.* the CD8 cytoplasmic domain), such that the protein is targeted to the cell membrane. Similarly, it may be desirable for the protein to comprise the extracellular sequences immediately juxtaposed with the cell membrane (*eg.* CD4 domains 3 and 4) in order to separate the CTLA-4-binding domain from the cell membrane. Synthetic linkers, such as glycine linkers, can be used for the same purpose.

The CTLA-4-binding domain of the protein preferably comprises an antibody with specificity for CTLA-4. This is preferably a single chain antibody (sFv). It is preferably specific for the CTLA-4 of a recipient organism.

In a typical embodiment, therefore, a protein of the second aspect can comprise a single chain antibody fused via a linker to the transmembrane and cytoplasmic domains of CD8.

The invention also provides nucleic acid which encodes a protein of the second aspect.

In addition, the invention provides a vector comprising said nucleic acid of the invention, and a cell transformed with said vector.

The invention also provides a delivery system comprising nucleic acid, and/or vector according to the second aspect of the invention, and means to deliver this material to a target cell.

Furthermore, the invention provides a cell which expresses a protein of the second aspect on its surface, preferably such that the protein can bind to available CTLA-4.

epithelial cells, which are B7-negative and have been shown to induce tolerance in rodent models of allogeneic transplantation.

The invention also provides a method of transplantation comprising the step of transplanting biological tissue according to the third aspect from a donor animal (*eg.* a pig) into a xenogeneic recipient animal (*eg.* a human).

In addition, the cells of the invention are suitable for pre-transplantation administration. This results in tolerance being induced in recipient T-cells before the xenograft itself is transplanted.

Furthermore, the invention provides a cell according to the third aspect for use as a medicament.

The invention also provides the use of a cell or of biological tissue according to the third aspect in the manufacture of a formulation for administering to a xenotransplant recipient.

The invention also provides the use of xenogeneic MHC class II, or nucleic acid encoding xenogeneic MHC class II, in the preparation of a formulation for administering to a xenotransplant donor.

Definitions

As used above, the term "nucleic acid" includes both DNA and RNA, although modified and synthetic nucleic acids are also included, For instance, the nucleic acid may be synthetic (*eg.* PNA), or may have modified inter-nucleotide linkages (*eg.* phosphorothioates). Furthermore, the term includes both sense and antisense nucleic acid sequences, as well as double-stranded sequences.

Preferably the nucleic acid comprises sequences suitable for the regulation of expression of protein according to the invention. This expression can preferably be controlled, such as cell-specific control, inducible control, or temporal control.

As used above, the term "vector" signifies a molecule which is capable of transferring nucleic acid to a host cell, and numerous suitable vectors are known in the art.

Preferably the vector is suitable for the production of a transgenic animal. Vectors suitable for the generation of transgenic pigs, for example, are described in references 13, 14, 15, 16 & 17.

As used above, the term "delivery system" refers to means for delivering genetic material to a target cell.

By expressing recipient MHC class II on the cells of the xenogeneic donor, the donor cells will present xenoantigens to a recipient T-cell in the context of self MHC class II. If the donor cells do not express B7, or if B7 is blocked, the xenoreactive recipient T-cell will not receive co-stimulatory signal 2 and will become anergic before the recipient's APCs have an opportunity to present the xenoantigens themselves.

The invention thus provides a cell which expresses on its surface MHC class II of a different organism. Preferably, this is a porcine cell expressing human MHC class II on its surface.

The MHC class II is preferably of the HLA-DR family.

The MHC class II is preferably constitutively expressed on the surface of the cells.

In order to prevent an allogeneic anti-MHC class II response, the MHC class II is preferably tissue-typed for maximum compatibility with the particular recipient. This will typically involve, for instance, ensuring that the HLA-DR expressed on the xenogeneic donor cell should match the HLA-DR of the particular recipient.

To ensure that xenoantigen display within the groove of the MHC class II molecule mirrors that found on professional APC, it is preferred that the cell should also express one or more of the following three proteins, each of which has an important role in antigen processing: invariant chain, HLA-DMα and HLA-DMβ.

The cell preferably does not express co-stimulatory molecules (*eg.* B7) on its surface. Typically, therefore, the donor cell is not a professional APC. It may, however, be a transfected non-immunogenic APC, such as an immature dendritic cell, which may be $B7^+$.

The invention also provides biological tissue comprising a cell according to the third aspect.

The invention further provides an animal comprising a cell and/or biological tissue according to the third aspect.

The invention also provides a process for rendering biological tissue suitable for xenotransplantation, comprising the step of treating said biological tissue such that it expresses xenogeneic MHC class II on the surface of its cells.

Preferably, this process comprises the steps of isolating non-immunogenic cells (*ie.* cells which cannot provide a co-stimulatory signal, such as B7-negative cells) from a xenogeneic organism and transfecting these cells with HLA-DR. The HLA-DR is preferably tissue-typed for a specific recipient. Furthermore, the cells may also be transfected with other proteins necessary for efficient antigen processing. Examples of suitable non-immunogenic cells include renal tubular

invention in order to produce a transgenic animal. For example, a vector according to the invention could be specifically delivered to the heart of a pig to produce biological tissue suitable for xenotransplantation.

Alternatively, the animal might be born as a transgenic animal. Many suitable approaches for generating such transgenic animals are known in the art [*eg*. refs. 18, 19, 20]. For example, direct manipulation of the zygote or early embryo, by microinjection of DNA for instance, is well known, as is the *in vitro* manipulation of pluripotent cells such as embryonic stem cells. Retroviral infection of early embryos has proved successful in a range of species, and adenoviral infection of zona-free eggs has been reported. Transgenesis and cloning of sheep by nuclear transfer has also been described (*eg*. WO97/07668).

Where the invention provides a process for rendering biological tissue suitable for xenotransplantation, said biological tissue may be so rendered either *in vivo* or *ex vivo*. For example, an animal organ may be *in vivo* transfected with a vector according to the invention, or an organ could be transfected *ex vivo* before transplantation or *in vivo* after transplantation.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described with reference to the attached drawings, in which:

Figure 1 illustrates the three aspects of the invention. "X" represents a xenogeneic cell (or, in the indirect activation pathway, a xenoantigen-presenting recipient APC), and "T" represents a recipient T-cell. In embodiment I, the delivery of co-stimulatory signal 2 is prevented by using a soluble form of CTLA-4. In embodiment II, anti-CTLA-4 is used to antagonise signal 2. In embodiment III, X expresses recipient MHC-II, but does not express B7.

Figure 2 shows the amino acid sequence of pCTLA-4 (SEQ ID NO:1). The following junctions are illustrated by a "*": signal peptide/extracellular domain; extracellular domain/transmembrane domain; transmembrane domain/cytoplasmic domain. An alignment with the human and bovine sequences is also shown. Homologies with pCTLA4 are:

Domain	Human	Bovine
Signal peptide	67.6%	86.5%
Extracellular domain	83.8%	84.6%
Transmembrane domain	96.1%	100%
Cytoplasmic domain	100%	100%
Overall	85.2%	89.2%

Certain vectors as described above may also function as suitable delivery systems. Likewise, certain delivery systems may also inherently be vectors, but this is not always the case. For instance, a viral vector can also function as a delivery system, whereas a liposomal delivery system is not a vector. The delivery system may be viral or non-viral. Non-viral systems, such as liposomes, avoid some of the difficulties associated with virus-based systems, such as the expense of scaled production, poor persistence of expression, and concerns about safety. Preferably the delivery system is suitable for use in gene therapy. Numerous appropriate delivery systems are known in the art.

Preferably, the delivery system will be targeted so that molecules according to the invention are taken up by cells suitable for xenotransplantation, or cells which have been transplanted. More preferably the delivery system will be specific for these cells. For example, the delivery system may be targeted to a specific organ, such as the heart or the kidney, or to a specific cell type, such as endothelial cells or professional APC.

To achieve this the delivery system may, for example, be a receptor-mediated delivery system, being targeted to receptors found on target cells. For example, the delivery system may be targeted to receptors found on heart cells, preferably to receptors found exclusively on heart cells, or it may be targeted to receptors found on endothelial cells, preferably to receptors found exclusively on endothelial cells.

The delivery system is preferably suitable for the generation of a transgenic animal. For example, the delivery system may be targeted to a gamete, a zygote, or an embryonic stem cell.

The vectors and delivery systems of the invention can be used to transfect cells to produce cells according to the invention. The transfection can occur *in vivo* or *ex vivo*.

The term "biological tissue" as used above includes collections of cells, tissues, and organs. Accordingly the definition includes, for example, fibroblasts, a cornea, nervous tissue, a heart, a liver, or a kidney.

Where the second and third aspects of the invention provide "an animal", said animal is preferably suitable for the production of organs for xenotransplantation and/or cells of the invention (*eg.* cells for pre-xenotransplant administration to xenograft recipients). Preferably the animal is a mammal, and more preferably it is a transgenic pig or a transgenic sheep.

The animal might be treated whilst alive such that it comprises transgenic biological tissue (*ie.* treated by gene therapy). Preferably, a live animal is transfected with a vector according to the

Figure 16 shows the sequence of cloned human CD8α. This differs from the GenBank sequence at positions 231 (T→G), 244 (A→G), 266 (T→C), and 437 (T→C).

Figure 17 shows the binding of human and murine CTLA4-Ig to IPEC, in order to define clones as B7-negative or B7-positive.

Figure 18 shows the binding to transfected cells of HLA-DR-specific mAb L243.

Figure 19 shows the proliferation by human T-cells to HLA-DR-1 transfected IPEC.

Figure 20 shows the results of a human T-cell proliferation assay following 2 days of incubation HLA-DR-1 transfected cells. The X-axis indicates the stimulator cells used in the second step of the proliferation assay. The black bars show results with CD4 T-cells which were primed with B7-positive transfectants; the white bars (hardly visible) show results after priming with B7-negative transfectants. The first graph shows results with cells harvested on day 3; the second graph shows results from a harvest on the sixth day.

Figure 21 shows the proliferation of an APC-dependent, HLA-DR-1 restricted T-cell line raised against IPEC. The stimulator population is indicated on the X-axis.

DESCRIPTION OF EMBODIMENTS

Soluble porcine CTLA-4

Porcine CTLA-4 ("pCTLA4") was cloned from PHA-activated pig T cells. RNA was prepared using standard techniques and pCTLA4 was amplified by PCR using primers:

5'-TTGAAGCTTAGCCATGGCTTGCTCTGGA-3' (5' primer)

5'-TAATGAATTCTCAATTGATGGGAATAAAATAAG-3' (3' primer)

The resulting 700bp fragment was sub-cloned into *EcoR*I/*Hind*III digested pBluescript, and the nucleotide sequence was determined using the standard T3 and T7 primers. The sequence of a single clone is shown in figure 3, which also shows a comparison with the human and bovine CTLA-4 sequences.

The predicted amino acid sequence of pCTLA4 is shown in figure 2, with a comparison with that of human and cattle. Of significance is the predicted amino acid difference at residue 97, which is important in B7 binding, being part of the conserved hexapeptide motif MYPPPY. In pCTLA4, residue 97 is leucine (giving LYPPPY), whereas other species have methionine (although leucine has also been found in bovine CD28 [21]). This important amino acid

Figure 3 shows a similar alignment, but at the nucleotide level. Homologies are as follows:

Domain	Human	Bovine
Signal peptide	76%	81.3%
Extracellular domain	85.2%	86.3%
Transmembrane domain	92.3%	96.2%
Cytoplasmic domain	96.5%	97.7%
Overall	86.1%	88.3%

Figure 4 shows the amino acid sequence of the pCTLA4-Ig construct. The underlined sequence shows the flexible linker GGSGGAA, which also denotes the junction between pCTLA4 and the IgG1 domains.

Figure 5 shows the results of flow cytometric analysis of hCTLA4-Ig (○ & □) and pCTLA4-Ig (◊ & Δ) binding to human fibroblasts transfected with either human B7 (lower two lines) or porcine B7 (upper two lines).

Figure 6 shows the selective inhibition of proliferation by pCTLA4-Ig (○ & Δ) compared to hCTLA4-Ig (□ & ◊) when co-stimulated by human B7 (□ & ○) or porcine B7 (◊ & Δ).

Figure 7 shows the inhibition of human CD4^{+} T cell proliferation by hCTLA4-Ig (□) or pCTLA4-Ig (◊) when human (7A) or porcine (7B) cells expressing MHC-class II were used as stimulators in a five day mixed leukocyte reaction.

Figure 8 shows the nucleotide sequence of an anti-human CTLA-4 sFv. The inferred protein sequence is shown in **Figure 9**. **Figure 10** shows the nucleotide sequences of four anti-murine CTLA-4 sFv. The inferred protein sequences are shown in **Figure 11**. The heavy and light chains are linked by a serine-glycine linker as indicated in Figures 9 and 11..

Figure 12 shows the construct encoding the soluble Ig-fusion of the CTLA-4-specific sFv.

Figure 13 shows the inhibition of T cell proliferation by cells expressing either an anti-hCTLA-4 sFv (□) or a control sFv (○).

Figure 14 shows construct encoding the membrane-bound form of the anti-CTLA-4 sFv.

Figure 15 shows (A) the nucleotide sequence and (B) the amino acid sequence of human CTLA-4. The start codon is underlined. At position –21, the sequence differs from GenBank sequence L15006, and at position 110 the sequence differs from both L15006 and M74363.

In the experiments using transfected fibroblast stimulators (expressing HLA class II and either human or pig B7), hCTLA4-Ig inhibited all proliferative responses (Figure 6, □ & ◊). In contrast, pCTLA4-Ig only fully inhibited the response when stimulators expressed porcine B7 (Δ); the proliferative response to cells expressing human B7 was only minimally affected (○).

In similar experiments, pCTLA4-Ig failed to have a significant inhibitory effect on the proliferative responses to human cells expressing MHC class II and human B7 but did inhibit the response to porcine stimulators (figure 7).

These results highlight the effective inhibitory properties of pCTLA4-Ig when T cell co-stimulatory signals are provided by porcine B7. The failure to prevent T cell proliferation when co-stimulation is mediated by human B7 also demonstrates species-specific action. It can be concluded that pCTAL4-Ig shows species-specific binding to and inhibition of the functional effects of porcine B7, but not human B7.

Properties of pCTLA-4-Ig

The binding characteristics of pCTLA4-Ig to both human and porcine B7-family molecules may be compared to those of hCTLA4-Ig, for example using the following tests:

(i) flow cytometric analysis of binding to porcine and human APC, and to transfectants expressing porcine or human B7 (see above)

(ii) quantitative characterisation of binding using Biacore™.

(iii) functional analysis of the effects of CTLA4-Ig on human anti-pig and human allogeneic mixed lymphocyte cultures.

(iv) functional assessment of the ability of pCTLA4-Ig to prolong porcine islet xenograft survival after transplantation into B6 mice.

A membrane-associated protein which binds to CTLA-4

A phage display library containing 10^{12} semi-synthetic variable sequences was screened using human or murine CTLA4-Ig and a control human IgG1 myeloma protein. The sFv from a phage displaying differential binding to the human CTLA4-Ig protein after 4 rounds of screening were isolated and purified using standard techniques. The nucleotide and inferred amino acid sequences are shown in figures 8, 9, 10, and 11.

The sFv were amplified by PCR using specific primers based on the nucleotide sequences. The distal portions of the primers were based on sequence within pHOOK1. The 5' primer

difference is believed to be of key importance to the advantageous differential binding of pCTLA4 to human and pig B7.

The extracellular domain of pCTLA4 was amplified using the 5' primer described above and

5'-CGGTTCTGCAGCACCACCGGAGCCACCATCAGAATCTGGGCATGGTTCTGGATCAATGAC-3'

This amplified from position 484, introduced an 18 base-pair segment encoding a linker GGSGGAA sequence (underlined), and introduced a *Pst*I site (bold) to allow in-frame ligation to the hinge region of human IgG1. The resulting 500bp fragment was sub-cloned into *Hind*III/*Pst*I digested pBluescript-IgG1 containing genomic DNA encoding intronic sequences and the hinge, CH2, CH3 and 3' untranslated exons of human IgG1 between *Pst*I/*Not*I sites. The amino acid sequence of the resulting soluble pCTLA4-Ig is shown in figure 4.

Expression of pCTLA4-Ig

The chimeric pCTLA4-Ig DNA sequence was released from pBluescript as a *Hind*III/*Bst*XI fragment and was sub-cloned into the expression vector pHOOK-3™ (Invitrogen). This was used to transfect DAP.3 or CHO-K1 cells using standard calcium phosphate precipitation . G418-resistant cells were separated using the CaptureTec™ system. These transfected cells were grown in tissue culture flasks until confluent, at which point the medium was changed, and the cells were kept in culture for a further 3 days. At this stage the medium was harvested and perfused through a protein G column. pCTLA-4-Ig was eluted under acid conditions. The concentration of the eluted protein was calculated using ELISA with an antibody directed against human IgG1 and a standard human IgG1 myeloma protein.

The binding characteristics of pCTLA4-Ig were compared to those of human CTLA4-Ig using flow cytometric analysis with human fibroblasts transfected with either human B7-1 or porcine B7-2. For these experiments, the concentration of pig and human CTLA4-Ig were equivalent as assessed by ELISA. As illustrated in figure 5, human and porcine CTLA4-Ig appeared to have similar binding characteristics on human cells expressing porcine B7. Unlike human CTLA4-Ig, however, pCTLA4-Ig failed to bind human B7, implying that pCTLA4-Ig binds preferentially to porcine B7 and is useful as a species-specific reagent.

pCTLA4-Ig was used to inhibit human T cell proliferative responses to a variety of stimulators. In these assays, co-stimulation of the T cell response was provided by either porcine or human B7, expressed either by transfection or naturally on professional APCs. These experiments are demonstrated in figures 6 and 7.

Membrane-associated CTLA-4 construct

The expression of CTLA-4 on by activated T-cells is only transient so, to test the functional characteristics of the anti-CTLA4-sFv, chimeric constructs comprising the DNA sequences encoding the extracellular domains of human or murine CTLA4 and the transmembrane/cytoplasmic sequences of human CD8 were made. Cells expressing these constructs can be used for the study of the anti-CTLA4-sFv protein.

RNA from PHA-activated human T cells was prepared using standard techniques. hCTLA4 was amplified PCR using primers:

5'-TTCAAAGCTTCAGGATCCTGAAAGGTTTTG-3' introducing a *Hind*III site (5' primer)

5'-TAATGAATTCTCAATTGATGGGAATAAAATAAG-3' introducing an *EcoR*I site (3' primer)

The resulting fragment was sub cloned into *Hind*III/*EcoR*I digested pBluescript and the nucleotide sequence determined using standard T3 and T7 primers. The sequence of a single clone is shown in figure 15. This differed by a single base (position 439) from GenBank-listed sequences for human CTLA-4. The predicted amino acid sequence of hCTLA4 is also shown.

The extracellular domain of hCTLA-4 was amplified using 5' primer described above and:

5'-GATGTAGATATCACAGGCGAA**GTCGAC**<u>ACCACCGGAGCCACC</u>AATTACATAAATCTGGGCTCCGTTGCCTATGCCC-3'

This amplified from position 457 and included a 15 base segment encoding a flexible GGSGG amino acid linker (underlined), along with a unique *Sal*I site (highlighted). The resulting fragment was sub cloned into *Hind*III/*Sal*I digested pBluescript and sequenced.

hCD8 was PCR-amplified from resting T-cells using primers:

5'-TCGCGCCCAAGCTTCGAGCCAAGCAGCGT-3' introducing a *Hind*III site (5' primer)

5'-TAATGAATTCTCAATTGATGGGAATAAAATAAG-3' introducing an *EcoR*I site (3' primer)

The resulting fragment was sub cloned into *Hind*III/*EcoR*I digested pBluescript and the nucleotide sequence determined using standard T3 and T7 primers. The sequence of a single clone is shown in figure 16. This clone differed from the sequence deposited with GenBank at four positions, although none of these were within the region that was subsequently amplified.

The transmembrane (TM) and cytoplasmic (C) domains of hCD8 were amplified using the 3' primer described above and the following 5' primer:

5'-CATAGGCAACGGAGCCCAGATTTATGTAATT<u>GGTGGCTCCGGTGGT</u>**GTCGAC**TTCGCCTGTGATATCTACATC-3'

contained an *Apa*I site and the 3' primer contained a *Sal*I site, both of which were predicted to be unique. The resulting sFv were sub-cloned into pBluescript for sequencing to determine faithful amplification. The *Apa*I/*Sal*I fragments were then sub-cloned into pHOOK1, where it is flanked upstream by an in-frame signal sequence from the murine Ig κ–chain and a haemaggluttinin A epitope sequence, and downstream by two in-frame *myc* sequences and a transmembrane sequence from the PDGF receptor.

The *myc* sequences from pHOOK1 were amplified by PCR using the 5' primer 5'-GAGCTGAAACGGGCGGCCGCAGAAC-3', which contains a *Not*I site (underlined) and the 3' primer 5'-CTGGCCTGCAGCATTCAGATCC-3', which introduced a *Pst*I site (underlined). The resulting 113 base pair fragment was sub-cloned into *Not*I/*Pst*I digested pBluescript.

The sFv was released from pHOOK1 as an *EcoR*I/*Not*I fragment, and was ligated into *EcoR*I/*Pst*I digested pBluescript-IgG1, along with the *Not*I/*Pst*I PCR product [Figure 12]. This construct encodes a soluble Ig-fusion of the CTLA-4-specific sFv. For expression in eukaryotic cells, the construct was sub-cloned into pHOOK3 as a *Hind*III/*BstX*I fragment.

To confirm cell-surface expression of the sFv, the pHOOK construct was transfected into cells already expressing HLA-DR molecules and human B7. Cells resistant to G418 or mycophenolic acid, depending on the vector used, were grown in culture. Cells expressing the anti-CTLA4-sFv construct on the cell surface were identified by flow cytometric analysis using hCTLA4-Ig. These cells were cloned by limiting dilution and were used as stimulators of T cell proliferation in 5 day cultures. The results of one experiment are shown in figure 13. Cells expressing the anti-hCTLA4 sFv failed to stimulate T cell proliferation (□), whereas those expressing a control sFv stimulated proliferation in the same way as normal cells (○).

In different experiments, the *EcoR*I/*Sal*I fragment of the construct shown in Figure 12 was co-ligated with the transmembrane and cytoplasmic domains of human CD8 (isolated as a *Sal*I/*BamH*I fragment from pBluescript-hCD8) into *EcoR*I/*BamH*I digested pBluescript [Figure 14].

The *EcoR*I/*BamH*I fragment from pBluescript was sub-cloned into the expression vector pHβApr-1-neo or the sister vector pHβApr-1-gpt. These were transfected into cells already expressing HLA-DR molecules and B7 and selected as described above for the pHOOK construct.

Surface expression of MHC class II on transfected IPEC cells was detected using monoclonal antibody L243 (specific for HLA-DR) [figure 18] or M5-114 (specific for murine MHC class II). MHC class II-positive cells underwent several rounds of fluorescence activated cell sorting before being cloned by limiting dilution.

A second batch of transfectants was prepared in exactly the same way, but with additional transfected cDNAs encoding HLA-DMA and HLA-DMB and p31Ii (invariant chain) in the expression vector pCMU.

Anergy induction in allogeneic T-cells by MHC class II-expressing cells

Human T-cells were purified using standard protocols [3]. For primary proliferation assays, T-cells were incubated for 5 days with fixed numbers of irradiated stimulator cells, before addition of 1μCi ^{3}H-thymidine sixteen hours prior to harvesting onto glass fibre filters. The filters were read in a scintillation counter.

B7-positive IPEC caused significant, anti-DR1 specific proliferative responses, whereas B7-negative IPEC failed to initiate any proliferative response [figure 19].

Two step anergy induction assays were established by a standard protocol [23]. In the primary, tolerance-induction step, T-cells incubated with B7-positive IPEC mounted an anti-DR1 proliferative response in the secondary step with the kinetics of a primed secondary immune response (maximal at three days). However, T-cells incubated with B7-negative IPEC in the primary step became tolerant to DR1 and failed to mount a response on subsequent exposure to DR1-expressed on B7-positive IPEC [figure 20].

Anergy induction in DR1-restricted T cells by DR1-expressing pig cells.

CD4$^+$ T-cells from a DR1-expressing individual were purified according to standard procedures. In primary proliferation assays, they proliferated to B7-positive IPEC transfected with HLA-DR1, indicating that the DR1 can perform as a restriction element for pig-peptide-specific T-cells. Assays comparing the proliferative response to B7-positive and B7-negative DR1+ transfectants are being performed.

Two step anergy induction assays may also be performed to demonstrate that DR1-transfected, B7-negative pig cells induce anergy in HLA-DR-restricted human T-cells.

This amplified from position 532 and included a 15 base segment encoding a flexible GGSGG amino acid linker (underlined), along with a unique *Sal*I site (highlighted). The resulting fragment was sub cloned into *Hind*III/*Sal*I digested pBluescript and called pBluescript-hCD8.

The extracellular domain of human CTLA-4 was cut from pBluescript as an *EcoR*I/*Sal*I fragment, and the TM-IC domain of CD8 cut as a *Sal*I/*BamH*I fragment. Together they were ligated back into *EcoR*I/*BamH*I digested pBluescript. The whole CTLA-4-CD8 chimera was then removed as a single *EcoR*I fragment and was sub-cloned into a number of expression vectors for expression into the human T cell leukaemia line J6.

Properties of the cell-surface anti-CTLA4 proteins

The cell-surface anti-CTLA-4 proteins may be further characterised by the following functional tests:

i) Flow cytometric assessment of the interaction between cells expressing the membrane-bound anti-CTLA4-sFv-CD8 protein and soluble human CTLA4-Ig.

ii) Quantitative assessment of the interaction between the soluble anti-CTLA4-sFv-Ig fusion protein and soluble human CTLA4-Ig, using Biacore™

iii) Analysis on the signalling events resulting from the binding of soluble human CTLA4-Ig to J6 transfectants expressing the anti-CTLA4-sFv-CD8 fusion protein.

iv) Analysis of T cell responses (*eg.* proliferation, cytokine production, anergy induction) when stimulation in an allogeneic mixed lymphocyte response is provided by an HLA-DR-positive, B7-positive, anti-CTLA4-sFv-CD8-positive cell line.

B7-negative porcine cells expressing murine MHC class II

Fifty cloned immortalised porcine aortic endothelial cells (PAEC) were generated from monolayers of PAEC by intranuclear microinjection with pZipSVU19 DNA [22]. From the immortalised cells (IPEC), B7-negative clones were identified by flow cytometric screening with hCTLA4-Ig and mCTLA4-Ig [see figure 17]. These were then transfected with cDNAs encoding HLA-DRA and DRB1*0101 in the plasmid expression vectors pcExV1-gpt and pHβApr-1neo, and cells were placed under selection with MXH and G418. For comparison, B7-positive IPEC controls were generated similarly [4].

Another series of IPEC transfectants expressing the murine MHC class II molecule I-A^b were also generated for experiments in mice.

REFERENCES (The contents of which are incorporated in full)

1 Squinto SP (1996) Xenogeneic organ transplantation. *Curr Opin Biotech* 7: 641-645.

2 McCurry *et al.* (1996) Human complement regulatory proteins expressed in transgenic swine protect swine xenografts from humoral injury. *Transplant Proc* 28: 758.

3 Dorling *et al.* (1996). Detection of primary direct and indirect human anti-porcine T cell responses using a porcine dendritic cell population. *Eur J Immunol* 26: 1378-1387.

4 Dorling *et al.* (1996) Cellular xenoresponses: Although vigorous, direct human T cell anti-pig primary xenoresponses are significantly weaker than equivalent alloresponses. *Xenotransplantation* 3: 149-157.

5 Auchincloss (1995) Why is cell-mediated xenograft rejection so strong? *Xeno* 3: 19.

6 Auchincloss (1988) Xenogeneic transplantation. *Transplantation* 46: 1.

7 Dorling *et al.* (1996) Cellular xenoresponses: Observation of significant primary indirect human T cell anti-pig xenoresponses using co-stimulator-deficient or SLA class II-negative porcine stimulators. *Xenotransplantation* 3: 112-119.

8 Linsley *et al.* (1991) CTLA-4 is a second receptor for the B-cell activation antigen B7. *J Exp Med* 174:561-569.

9 Lane *et al.* (1993) Expression and functional properties of mouse B7/BB1 using a fusion protein between mouse CTLA4 and human γ1. *Immunology* 80: 56.

10 Cohen (1992) Mounting a targeted strike on unwanted immune responses. *Science* 257:751.

11 Baliga *et al.* (1994) CTLA4Ig prolongs allograft survival while suppressing cell mediated immunity. *Transplantation* 58: 1082-1090.

12 Lenschow *et al.* (1992) Long term survival of xenogeneic pancreatic islet grafts induced by CTLA4-Ig. *Science* 257: 789-792.

Overlap between the pig peptides processed by professional human APC for presentation on HLA-DR and those presented on MHC class II of IPEC transfected with HLA-DR.

A human T-cell line against wild type IPEC was raised from human PBMC. The proliferative response of this line was dependent on the presence of human APC and inhibitable by antibodies against HLA-DR, indicating that the line had indirect specificity for processed porcine xenoantigens presented by human APC.

This line proliferated against B7-positive HLA-DR1-transfected IPEC [figure 21] implying that at least some of the processed pig peptides presented indirectly by professional human APC are also presented by transfected pig cells.

Studies in pig-islets-to-mouse model

In vivo, porcine pancreatic islet cells may be transplanted under the kidney capsule of streptozotocin-treated diabetic mice. Islet xenografts, being non vascular, are rejected solely by T-cells. Porcine islets are prepared from the pancreas of pigs under terminal anaesthesia, and their survival in the recipients assessed by maintenance of normoglycaemia. Mice are injected intravenously with B7-negative, I-A^b-expressing IPEC before transplantation of pig islets. This strategy can be combined with other aspects of the invention to tolerise the direct pathway of T-cell recognition, to ensure that rejection via the direct pathway does not occur. To assess whether a particular strategy has induced specific T-cell tolerance, nephrectomy of the islet-carrying kidney is performed before re-transplantation (under the capsule of the surviving kidney), of identical or third party porcine islets.

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

CLAIMS

1. A biological reagent capable of inhibiting T-cell mediated rejection of a xenotransplanted organ by blocking the delivery of co-stimulatory signal 2 in order to prevent the activation of xenoreactive T-cells in the recipient.

2. A method for inhibiting T-cell mediated rejection of a xenotransplanted organ, comprising blocking the delivery of co-stimulatory signal 2 in order to prevent the activation of xenoreactive T-cells in the recipient.

3. A method according to claim 2, comprising the administration to said recipient to a soluble form of the CTLA-4 protein from the xenogeneic donor organism.

4. A method according to claim 3, wherein said soluble protein comprises the extracellular domain of porcine CTLA-4 fused to a human $C\gamma 1$ sequence.

5. A soluble form of xenogeneic CTLA-4 for use as a medicament.

6. A protein comprising the amino acid sequence SEQ ID:1

7. Nucleic acid which encodes the protein according to claim 6

8. A biological reagent according to claim 1, wherein said reagent is a membrane-associated protein which can bind to CTLA-4.

9. A protein according to claim 8, comprising a single chain antibody with specificity for CTLA-4.

10. Nucleic acid which encodes a protein according to claim 8 or claim 9.

11. A cell which expresses a protein according to claim 8 or claim 9 on its surface.

12. Biological tissue comprising a cell according to claim 11.

13. An animal comprising a cell according to claim 11 and/or biological tissue according to claim 12.

14. A method of transplantation comprising the step of transplanting biological tissue according to claim 12 from a donor animal into a xenogeneic recipient animal.

13 Heckl-Östreicher *et al.* (1995) Functional activity of the membrane-associated complement inhibitor CD59 in a pig-to-human *in vtiro* model for hyperacture xenograft rejection. *Clin. Exp. Immunol.* 102:589-595.

14 McCurry *et al.* (1996) Human complement regulatory proteins expressed in transgenic swine protect swine xenografts from humoral injury. *Transplant Proc* 28: 758.

15 White *et al.* (1995) The control of hyperacute rejection by genetic engineering of the donor species. *Eye* 9: 185-189.

16 Yannoutsos *et al.* (1995) Production of pigs transgenic for human regulators of complement activation. *Transplant Proc* 27: 324-325.

17 Langford *et al.* (1996) Production of pigs transgenic for human regulators of complement activation using YAC technology. *Transplant Proc* 28: 862-863.

18 Bradley & Liu (1996) Target practice in transgenics. *Nature Genet* 14: 121-123.

19 Clarke (1996) The adenovirus and the egg: a new approach to transgenesis. *Nature Biotech.* 14:942.

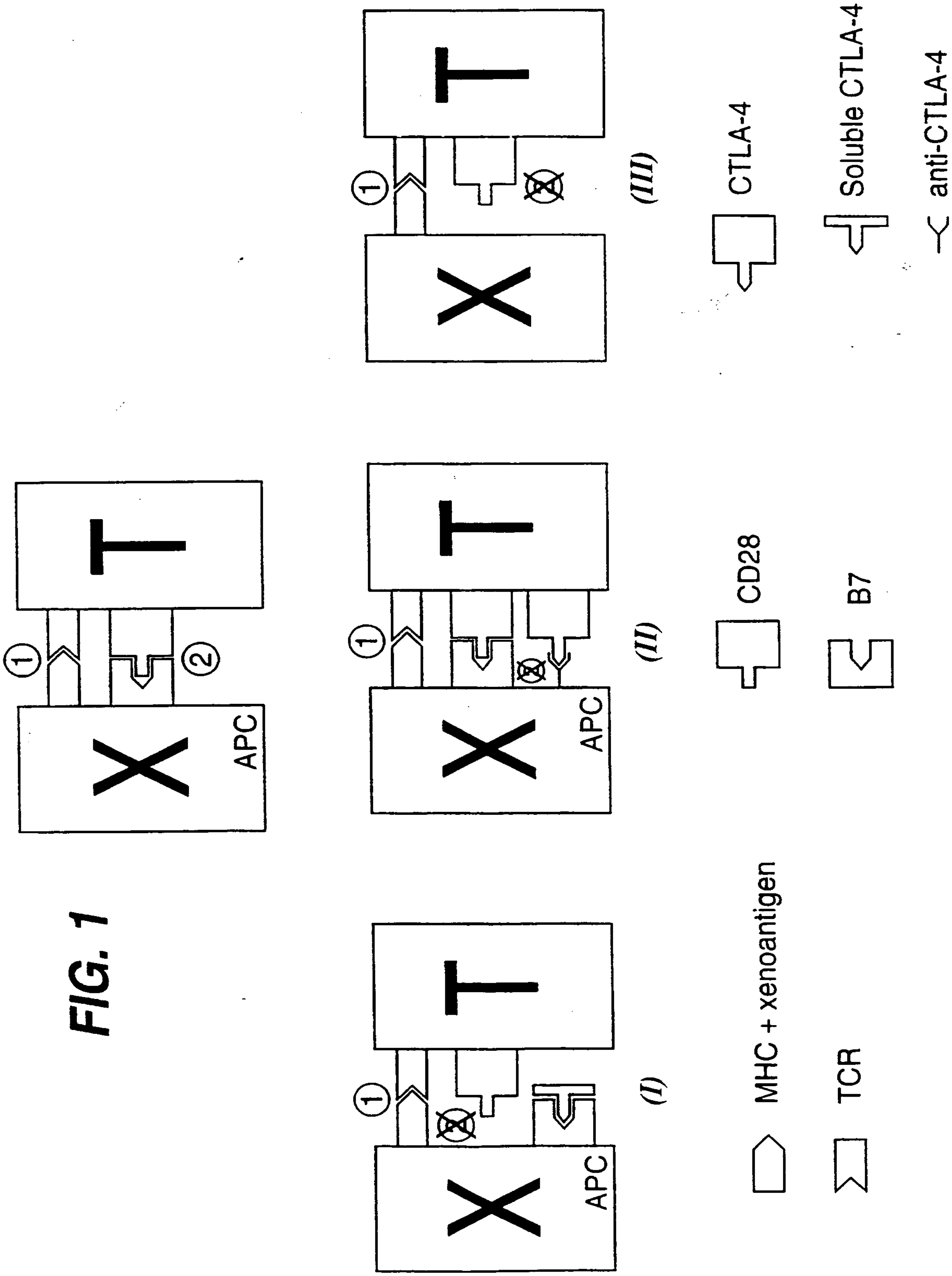
20 Wheeler (1994) Development and validation of swine embryonic stem cells: a review. *Reprod Fertil Dev* 6:563-568.

21 Parsons *et al.* (1996) Cattle CTLA-4, CD28 and chicken CD28 bind CD86: MYPPPY is not conserved in cattle CD28. *Immunogenetics* 43: 388-391.

22 Dorling *et al.* (1996) *In vitro* accommodation of immortalized porcine endothelial cells. *Transplantation* 62:1127-1136.

23 Marelli-Berg *et al.* (1996) Major histocompatibility complex class II-expressing endothelial cells induce allospecific nonresponsiveness in naive T cells. *J Exp Med* 183:1603.

FIG. 1


X
APC
T
1
2
(I)
(II)
(III)
MHC + xenoantigen
TCR
CD28
B7
CTLA-4
Soluble CTLA-4
anti-CTLA-4

15. A process for rendering biological tissue suitable for xenotransplantation, comprising the step of treating said biological tissue such that it expresses a protein according to claim 8 or claim 9 on the surface of its cells.

16. A protein according to claim 8 or claim 9, or nucleic acid according to claim 10, for use as a medicament.

17. The use of a protein according to claim 8 or claim 9, or of nucleic acid according to claim 10, in the preparation of a formulation for administration to a xenotransplant recipient or donor.

18. A biological reagent according to claim 1, wherein said reagent is a cell which expresses on its surface MHC class II of a different organism.

19. A cell according to claim 18, wherein said cell is a porcine cell expressing human MHC class II on its surface.

20. A cell according to claim 18 or claim 19, wherein said cell does not express B7 on its surface.

21. A cell according to claim 18 or claim 19, wherein said cell is a transfected immature dendritic cell

22. Biological tissue comprising a cell according to any one of claims 18, 19, 20 or 21.

23. An animal comprising biological tissue according to claim 22.

24. A method of transplantation comprising the step of transplanting biological tissue according to claim 22 from a donor animal into a xenogeneic recipient animal.

25. A process for rendering biological tissue suitable for xenotransplantation, comprising the step of treating said biological tissue such that it expresses xenogeneic MHC class II on the surface of its cells.

26. A cell according to any one of claims 18, 19, 20 or 21, for use as a medicament.

27. The use of biological tissue according to claim 22 in the manufacture of a formulation for administering to a xenotransplant recipient.

28. The use of xenogeneic MHC class II, or nucleic acid encoding xenogeneic MHC class II, in the preparation of a formulation for administering to a xenotransplant donor.

FIG. 3

```
1          11         21         31         41         51
ATGGCTTGCT CTGGATTCCG GAGCCATGGG GCTTGGCTGG AGCTTACTTC TAGGACCTGG
•••••••••C T••••••T•A •C•G••CAA• •••CA••••A •C••GG••G• C•••••••••
•••••••••• •••••••••A •••T•••••• A•••••-••• •-----•A•• ••••••••••

61         71         81         91         101        111
CCCTGTACAG CTCTGTTTTC TCTTCTCTTC ATCCCTGTCT TCTCCAAAGG GATGCACGTG
•••••C••TC TC•••••••T •••••••••• •••••••••• •••G•••••C A•••••••••
•••••C••T• •C••A••••T ••••G••••• ••••••••T• ••••T••••• ••••A•T•••
                                                        *
121        131        141        151        161        171
GCCCAACCTG CAGTAGTGCT GGCCAACAGC CGGGGTGTTG CCAGCTTTGT GTGTGAGTAT
•••••G•••• •T••G••A•• •••••G•••• ••A••CA•C• •••••••••• ••••••••••
A••••G•••C ••••G••••• •••T•G•••• •••••••••• •••••••CTC A•••••A•••
     §          §
181        191        201        211        221        231
GGGTCTGCAG GCAAAGCTGC CGAGGTCCGG GTGACAGTGC TGCGGCGGGC CGGCAGCCAG
•CA•••C••• •••••••CA• T••••••••• •••••••••• •T••••A••• T•A•••••••
•A••••T••• •••••••••A •••••••••• •••••••••• •••••GA••• A•••••••••

241        251        261        271        281        291
ATGACTGAAG TCTGTGCCGC GACATATACT GTGGAGGATG AGTTGACCTT CCTTGATGAC
G••••••••• •••••••G•• A••C••C•TG A•••G•A••• •••••••••• •••A•••••T
G••••C•••• •••••••T•G •••C••C•TG •••••••••• ••C•A••••• •••G•••••T

301        311        321        331        341        351
TCTACATGCA CTGGCACCTC CACCGAAAAC AAAGTGAACC TCACCATCCA AGGGCTGAGA
••C•TC•••• •G•••••••• ••GT•G•••T C••••••••• ••••T••••• •••A•••••G
-----------------------------------------------------------------
```

FIG. 2

```
       -30        -20        -10          1         11         21
MACSGFRSHG AWLELTSRTW PCTALFSLLF IPVFSKGMHV AQPAVVLANS RGVASFVCEY
•••L••QR•K •Q•N•AA••• •••L••F••• ••••C•A••• ••••••••S• ••I•••••••
••••••Q••• T•W--••••• ••••••F•V• ••••••••N• T••P••••S• ••••••S•••
                                         *

        31         41         51         61         71         81
GSAGKAAEVR VTVLRRAGSQ MTEVCAATYT VEDELTFLDD STCTGTSTEN KVNLTIQGLR
A•P•••T••• •••••Q•D•• V••••••••M MGN••••••• •I•••••SG• Q•••••••••
E•S•••D••• •••••E•••• V•••••G••M •••••••••• •••I•••RG• ••••••••••

        91        101        111        121        131        141
AVDTGLYICK VELLYPPPYY VGMGNGTQIY VIDPEPCPDS DFLLWILAAV SSGLFFYSFL
•M•••••••• •••MYPPPYY L•I•••A••• •••••••••• •••••••••• ••••••••••
•M•••••V•• •••MYPPPYY ••I••••••• •••••••••• •••••••••• ••••••••••
                                              *

       151        161        171        181
ITAVSLSKML KKRSPLTTGV YVKMPPTEPE CEKQFQPYFI PIN   SEQ ID:1 (pCTLA4)
L••••••••• •••••••••• •••••••••• •••••••••• •••   Human CTLA4
•••••••••• •••••••••• •••••••••• •••••••••• •••   Cattle CTLA4
      *
```

FIG. 4

```
     -30        -20        -10          1         11         21
MACSGFRSHG AWLELTSRTW PCTALFSLLF IPVFSKGMHV AQPAVVLANS RGVASFVCEY

      31         41         51         61         71         81
GSAGKAAEVR VTVLRRAGSQ MTEVCAATYT VEDELTFLDD STCTGTSTEN KVNLTIQGLR

      91        101        111        121        131        141
AVDTGLYICK VELLYPPPYY VGMGNGTQIY VIDPEPCPDS DGGSGGAAEP KSCDKTHTCP

     151        161        171        181        191        201
PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA

     211        221        231        241        251        261
KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ

     271        281        291        301        311        321
VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY

     331        341        351        361
SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
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------------------------------------------------------------------

361        371        381        391        401        411
GCCGTGGACA CTGGGCTCTA CATCTGCAAG GTGGAGCTCC TGTACCCACC ACCCTACTAT
•••A•••••• •G••A••••• •••••••••• •••••••••A •••••••••• G••A•••••C
•••A•••••• •••••••••• TG•••••••A •••••••••A •••••••G•• G••••••••C

421        431        441        451        461        471
GTGGGTATGG GCAACGGGAC CCAGATTTAT GTCATTGATC CAGAACCATG CCCAGATTCT
C••••C••A• •••••••AG• •••••••••• ••A••••••• •••••••G•• ••••••••••
•••••C••C• ••••T••A•• •••••••••C •••••••••• •••••••••• •••G••••••

481        491        501        511        521        531
GATTTCCTGC TCTGGATCCT GGCAGCAGTT AGTTCAGGGT TGTTTTTTTA CAGCTTCCTC
••C•••••C• •••••••••• T••••••••• •••••G•••• •••••••••• T•••••T•••
•••••T••C• •••••••••• •••••••••• •••••••••• •••••••C•• ••••••••••
  *
541        551        561        571        581        591
ATCACAGCTG TTTCTTTGAG CAAAATGCTA AAGAAAAGAA GTCCTCTTAC TACAGGGGTC
C••••••••• •••••••••• •••••••••• •••••••••• •C•••••••• A•••••••••
•••••••••• •••••••••• •••••••••• •••••••••• •C•••••••• ••••••••••
                        *
601        611        621        631        641        651
TATGTGAAAA TGCCCCCGAC AGAGCCAGAA TGTGAAAAGC AATTTCAGCC TTATTTTATT
•••••••••• •••••••A•• •••••••••• •••••••••• •••••••••• ••••••••••
•••••••••• •••••••A•• •••••••••• •••••••••• •••••••••• ••••••••••

661        671
CCCATCAATT GA               SEQ ID: 2 (pCTLA4)
•••••••••• ••               Human CTLA4
•••••••••• ••               Cattle CTLA4
```

FIG. 3 (CONTD.)

FIG. 7(A)

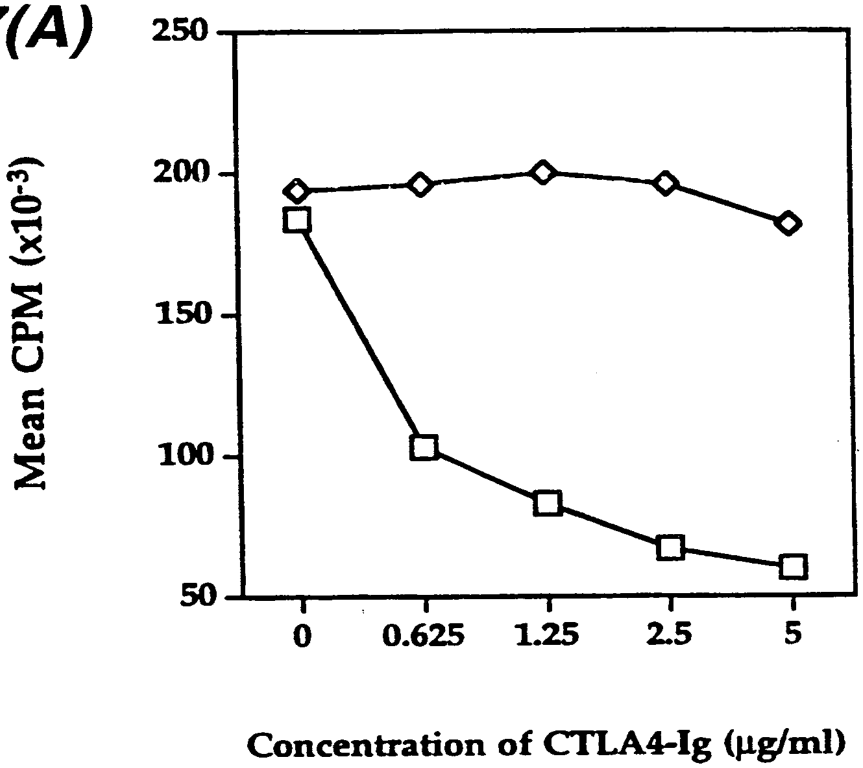

Mean CPM (x10^{-3})
250
200
150
100
50
0
0.625
1.25
2.5
5
Concentration of CTLA4-Ig (µg/ml)

FIG. 7(B)

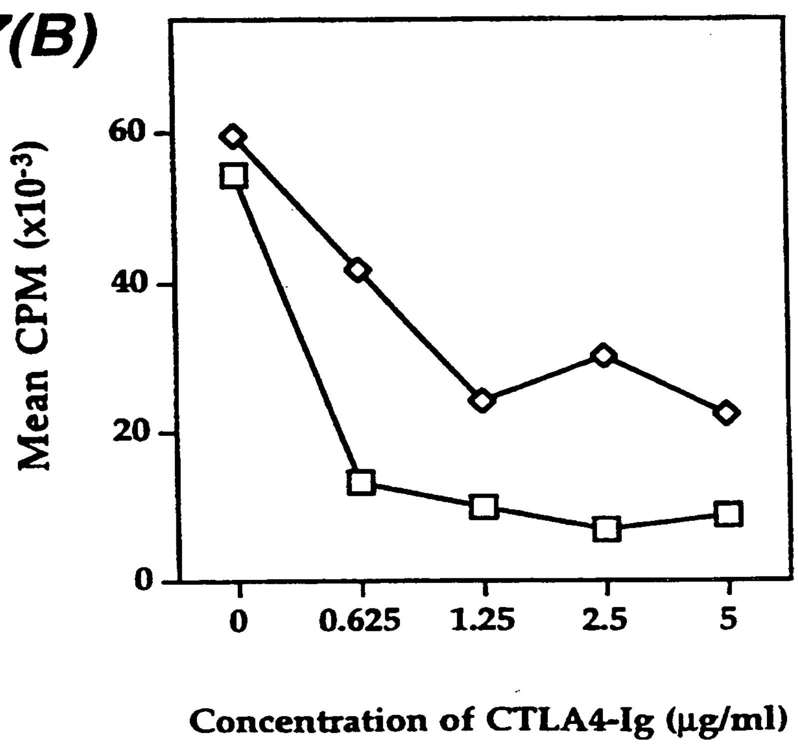

Mean CPM (x10^{-3})
60
40
20
0
0
0.625
1.25
2.5
5
Concentration of CTLA4-Ig (µg/ml)

FIG. 5

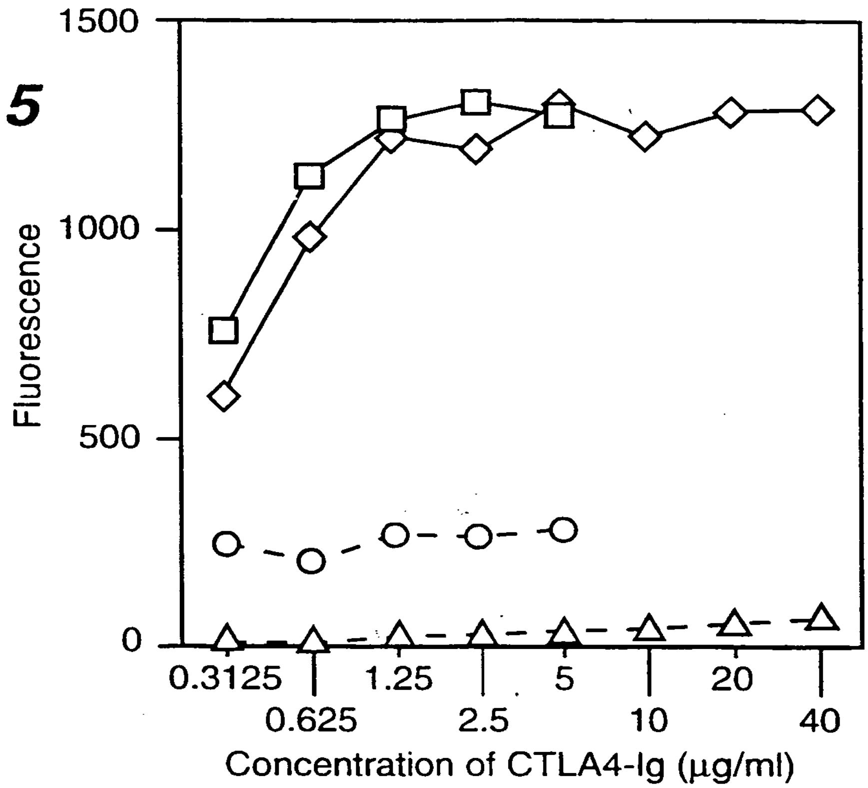

1500
1000
500
0
Fluorescence
0.3125
0.625
1.25
2.5
5
10
20
40
Concentration of CTLA4-Ig (µg/ml)

FIG. 6

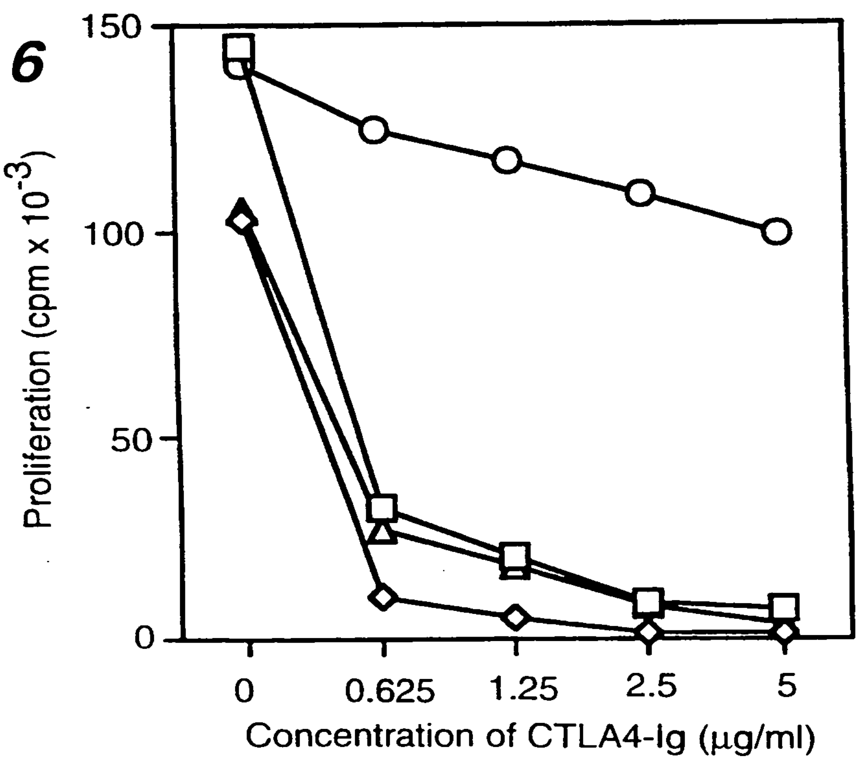

150
100
50
0
Proliferation (cpm x 10-3)
0
0.625
1.25
2.5
5
Concentration of CTLA4-Ig (µg/ml)

FIG. 9

```
1......... 11........ 21........ 31........ 41........ 51........ 61........ 71........
EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYAMSWVRQA PGKGLEWVSA ISGSGGSTYY ADSVKGRFTI SRDNSKNTLY

81........ 91........ 101....... 111....... 121....... 131....... 141....... 151.......
LQMNSLRAED TAVYYCARAG RILFDYWGQG TLVTVSSGGG GSGGGGSGGS ALQSVLTQPP SASGTPGQRV TISCSGSSSN
                                      LINKER

161....... 171....... 181....... 191....... 201....... 211....... 221....... 231.......
IGSNYVYWYQ QLPGTAPKLL IYRNNQRPSG VPDRFSGSKS GTSASLAISG LRSEDEADYY CAAWDDSLVF GGGTKLTVLG
```

FIG. 13

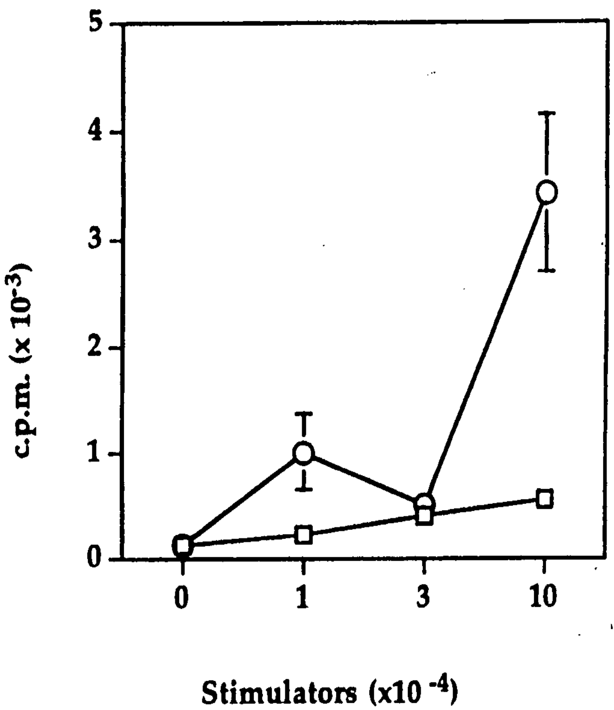


FIG. 8

```
1......... 11........ 21........ 31........ 41........ 51........ 61........ 71........
CCGAGGTGCA GCTGGTGGAG TCTGGGGGAG GCTTGGTACA GCCTGGGGGG TCCCTGAGAC TCTCCTGTGC AGCCTCTGGA

81........ 91........ 101....... 111....... 121....... 131....... 141....... 151.......
TTCACCTTTA GCAGCTATGC CATGAGCTGG GTCCGCCAGG CTCCAGGGAA GGGGCTGGAG TGGGTCTCAG CTATTAGTGG

161....... 171....... 181....... 191....... 201....... 211....... 221....... 231.......
TAGTGGTGGT AGCACATACT ACGCAGACTC CGTGAAGGGC CGGTTCACCA TCTCCAGAGA CAATTCCAAG AACACGCTGT

241....... 251....... 261....... 271....... 281....... 291....... 301....... 311.......
ATCTGCAAAT GAACAGCCTG AGAGCCGAGG ACACGGCCGT GTATTACTGT GCAAGAGCTG GTCGTATTTT GTTTGACTAT

321....... 331....... 341....... 351....... 361....... 371....... 381....... 391.......
TGGGGCCAAG GTACCCTGGT CACCGTCTCG AGTGGTGGAG GCGGTTCAGG CGGAGGTGGC TCTGGCGGTA GTGCACTTCA

401....... 411....... 421....... 431....... 441....... 451....... 461....... 471.......
GTCTGTGCTG ACTCAGCCAC CCTCAGCGTC TGGGACCCCC GGGCAGAGGG TCACCATCTC TTGTTCTGGA AGCAGCTCCA

481....... 491....... 501....... 511....... 521....... 531....... 541....... 551.......
ACATCGGAAG TAATTATGTA TACTGGTACC AGCAGCTCCC AGGAACGGCC CCCAAACTCC TCATCTATAG GAATAATCAG

561....... 571....... 581....... 591....... 601....... 611....... 621....... 631.......
CGGCCCTCAG GGGTCCCTGA CCGATTCTCT GGCTCCAAGT CTGGCACCTC AGCCTCCCTG GCCATCAGTG GGCTCCGGTC

641....... 651....... 661....... 671....... 681....... 691....... 701....... 711.......
CGAGGATGAG GCTGATTATT ACTGTGCAGC ATGGGATGAC AGCCTGGTAT TCGGCGGAGG GACCAAGCTG ACCGTCCTAG

721
GT
```

FIG. 10

```
                  10        20        30        40        50        60
                   |         |         |         |         |         |
M1 sFv    CATGG-CCGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAG
M3 sFv    CATGG-CCCAGGTGCAGCTGCAGGAGTCGGGCCCAGGACTGGTGAAGCCTTCGGAGACCCTGTC
M19 sFv   CATGG-CCCAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAGGCCTGGGGCCTCAGTGAA
M24 sFv   CATGGGCCCAGGTGCAGCTGTTGCAGTCTGCAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAA

              70        80        90       100       110       120
               |         |         |         |         |         |
M1 sFv    ACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGC--TA--TGCCAT--GAGCTGGGTCCGC
M3 sFv    CCTCACCTGCACTGTCTCTGGTGGCTCCGTCAGCAGTGGTAGTTACTACTGGAGCTGGATCCGG
M19 sFv   GGTTTCCTGCAAGGCATCTGGATACACCTTCACCAGC------TACTATATGCACTGGGTGCGA
M24 sFv   GATCTCCTGTAAGGGTTCTGGATACAGCTTTACCAGC------TACTGGATCGGCTGGGTGCGC

         130       140       150       160       170       180       190
           |         |         |         |         |         |         |
M1 sFv    CAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACT
M3 sFv    CAGCCCCCAGGGAAGGGACTGGAGTGGATT--GGGTAT-ATCTATTACAGTGGGAGCACCAACT
M19 sFv   CAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAATAATCAACCCTAGTGGTGGTAGCACAAGCT
M24 sFv   CAGATGCCCGGGAAAGGCCTGGAGTGGATGGGGATCATCTATCCTGGTGACTCTGATACCAGAT

               200       210       220       230       240       250
                 |         |         |         |         |         |
M1 sFv    ACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCT
M3 sFv    ACAACCCCTCCCTCAAGAGTCGAGTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCT
M19 sFv   ACGCACAGAAGTTCCAGGGCAGAGTCACCATGACCAGGGACACGTCCACGAGCACAGTCTACAT
M24 sFv   ACAGCCCGTCCTTCCAAGGCCAGGTCACCATCTCAGCCGACAAGTCCATCAGCACCGCCTACCT

           260       270       280       290       300       310       320
             |         |         |         |         |         |         |
M1 sFv    GCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCAAGAGCTG------GT
M3 sFv    GAAGCTGAGCTCTGTGACCGCTGCGGACACGGCCGTGTATTACTGTGCAAGAATGC------GG
M19 sFv   GGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTGCAAGAGTGGCTCCCTAT
M24 sFv   GCAGTGGAGCAGCCTGAAGGCCTCGGACACGGCCGTGTATTACTGTGCAAGATT--TTCGCT-T

                   330       340       350       360       370       380
                     |         |         |         |         |         |
M1 sFv    CGTATTTTGTTTGACTATTGGGGCCAAGGTACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTT
M3 sFv    AAGGATAAGTTTGACTATTGGGGCCAAGGTACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTT
M19 sFv   GTGAATACGCTTGTTTTTTGGGGCCAAGGTACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTT
M24 sFv   GGTGGT---TTTGACTATTGGGGCCAAGGTACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTT
```

```
                 390       400       410       420       430       440
                   |         |         |         |         |         |
M1 sFv   CAGGCGGAGGTGGCTCTGGCGGTAGTGCACTTCAGTCTGTGCTGACTCAG---CCACCCTCAGC
M3 sFv   CAGGCGGAGGTGGCTCTGGCGGTAGTGCACTTCAGTCTGTGCTGACTCAG---CCACCCTCAGC
M19 sFv  CAGGCGGAGGTGGCTCTGGCGGTAGTGCACTTTCTTCTGAGCTGACTCAG---GACCCTGCTGT
M24 sFv  CAGGCGGAGGTGGCTCTGGCGGTAGTGCACTTGACATCCAGTTGACCCAGTCTCCATCCTTCCT

           450       460       470       480       490       500       510
             |         |         |         |         |         |         |
M1 sFv   GTCTGGGACCCCCGGGCAGAGGGTCACCATCTCTTGTTCTGGAAGCAGCTCCAACATCGGAAGT
M3 sFv   GTCTGGGACCCCCGGGCAGAGGGTCACCATCTCTTGTTCTGGAAGCAGCTCCAACATCGGAAGT
M19 sFv  GTCTGTGGCCTTGGGACAGACAGTCAGGATCACATGCCAAGGAGACAGCCTCA------GAAGC
M24 sFv  GTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGCC---GGGCCAGTCAGGGCATT---AGC

                 520       530       540       550       560       570
                   |         |         |         |         |         |
M1 sFv   AATTATGTATACTGGTACCAGCAGCTCCCAGGAACGGCCCCCAAACTCCTCATCTATAGGAATA
M3 sFv   AATTATGTATACTGGTACCAGCAGCTCCCAGGAACGGCCCCCAAACTCCTCATCTATAGGAATA
M19 sFv  TATTATGCAAGCTGGTACCAGCAGAAGCCAGGACAGGCCCCTGTACTTGTCATCTATGGTAAAA
M24 sFv  AGTTATTTAGCCTGGTATCAGCAAAAACCAGGGAAAGCCCCTAAGCTCCTGGTCTATGCTGCAT

             580       590       600       610       620       630       640
               |         |         |         |         |         |         |
M1 sFv   ATCAGCGGCCCTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCT
M3 sFv   ATCAGCGGCCCTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCACCTCAGCCTCCCT
M19 sFv  ACAACCGGCCCTCAGGGATCCCAGACCGATTCTCTGGCTCCAGCTCAGGAAACACAGCTTCCTT
M24 sFv  CCACTTTGCAAAGTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATCTGGGACAGAATTCACTCT

                   650       660       670       680       690       700
                     |         |         |         |         |         |
M1 sFv   GGCCATCAGTGGGCTCCGGTCCGAGGATGAGGCTGATTATTACTGTGCAGCATGGGATGACAGC
M3 sFv   GGCCATCAGTGGGCTCCGGTCCGAGGATGAGGCTGATTATTACTGTGCAGCATGGGATGACAGC
M19 sFv  GACCATCACTGGGGCTCAGGCGGAAGATGAGGCTGACTATTACTGTAACTCCCGGGACAGCAGT
M24 sFv  CACAATCAGCAGCCTGCAGCCTGAAGATTTTGCAACTTATTACTGTCA---ACAGCTTAATAGT

               710       720       730       740       750
                 |         |         |         |         |
M1 sFv   --CTG----GTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGTGC
M3 sFv   --CTGT-TTGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGTGCGGCCGC
M19 sFv  GGTTTTACTGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGTGC
M24 sFv  TACCGCTTGACGTTCGGCCAAGGGACCAAGCTGGAAATC--AAACGTG----C
```

FIG. 10 (CONTD.)

FIG. 11

```
                       10        20        30        40        50        60
M1 sFv Peptide    --EVQLVESGGGLVQPGGSLRLSCAASG--FTFSSYAMSWVRQAPGKGLEWVSAISGSGG
M3 sFv Peptide    MAQVQLQESGPGLVKPSETLSLTCTVSGGSVSSGSYYWSWIRQPPGKGLEWIGYIY-YSG
M19 sFv Peptide   --QVQLVQSGAEVKRPGASVKVSCKASG--YTFTSYYMHWVRQAPGQGLEWMGIINPSGG
M24 sFv peptide   --QVQLLQSAAEVKKPGESLKISCKGSG--YSFTSYWIGWVRQMPGKGLEWMGIIYPGDS

                       70        80        90       100       110       120
M1 sFv Peptide    STYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARAGR--ILFDYWGQGTLVT
M3 sFv Peptide    STNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARMRK--DKFDYWGQGTLVT
M19 sFv Peptide   STSYAQKFQGRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARVAPYVNTLVFWGQGTLVT
M24 sFv peptide   DTRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAVYYCARFS--LGGFDYWGQGTLVT

                      130       140       150       160       170       180
M1 sFv Peptide    VSSGGGGSGGGGSGGSALQSVLTQPPS-ASGTPGQRVTISCSGSSSNIGSNYVYWYQQLP
M3 sFv Peptide    VSSGGGGSGGGGSGGSALQSVLTQPPS-ASGTPGQRVTISCSGSSSNIGSNYVYWYQQLP
M19 sFv Peptide   VSSGGGGSGGGGSGGSALSSELTQDPA-VSVALGQTVRITCQGDS--LRSYYASWYQQKP
M24 sFv peptide   VSSGGGGSGGGGSGGSALDIQLTQSPSFLSASVGDRVTITCRASQG--ISSYLAWYQQKP

                      190       200       210       220       230       240
M1 sFv Peptide    GTAPKLLIYRNNQRPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSL--VFG
M3 sFv Peptide    GTAPKLLIYRNNQRPSGVPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSLF-VFG
M19 sFv Peptide   GQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSRDSSGFTVFG
M24 sFv peptide   GKAPKLLVYAASTLQSGVPSRFSGSGSGTEFTLTISSLQPEDFATYYCQQLNSYRLT-FG

M1 sFv Peptide    GGTKLTVLG
M3 sFv Peptide    GGTKLTVLGAA
M19 sFv Peptide   GGTKLTVLG
M24 sFv peptide   QGTKLEI--KR
```

FIG. 12

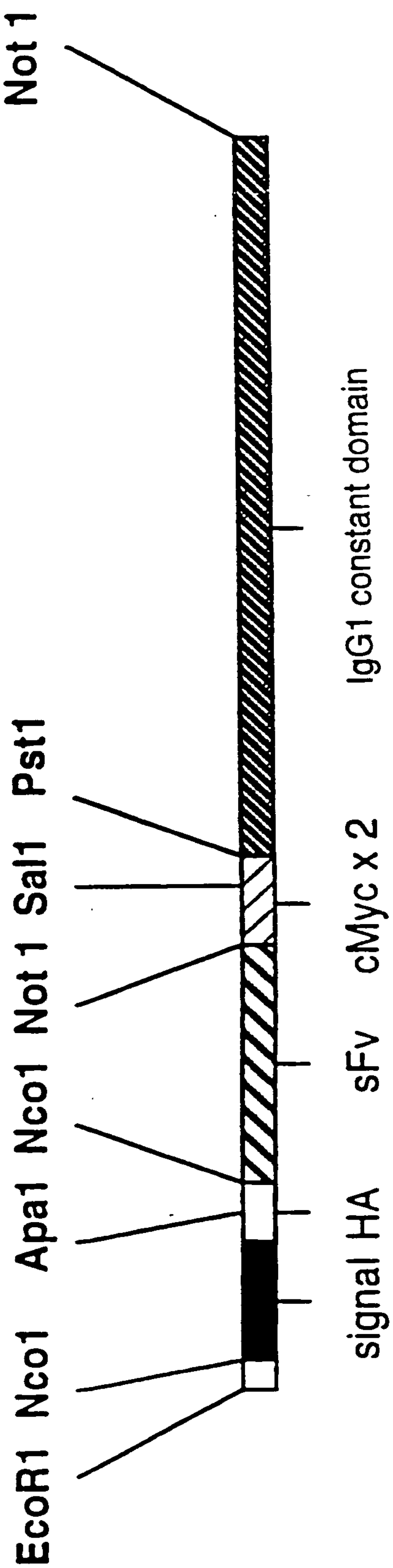

EcoR1
Nco1
Apa1
Nco1
Not 1
Sal1
Pst1
Not 1
signal HA
sFv
cMyc x 2
IgG1 constant domain

FIG. 14

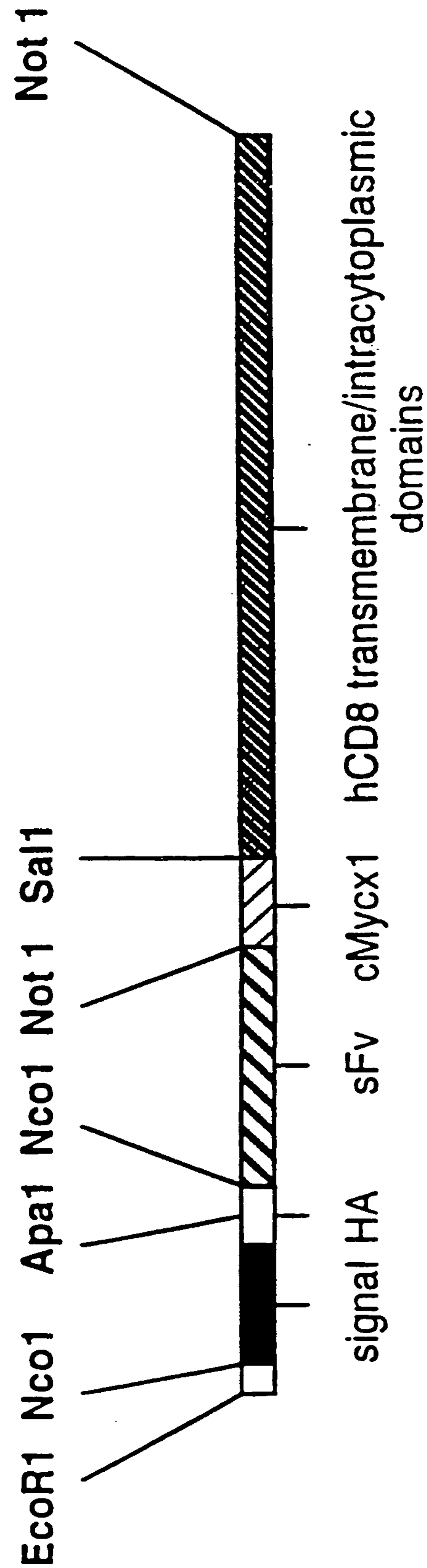

EcoR1
Nco1
Apa1
Nco1
Not 1
Sal1
Not 1
signal HA
sFv
cMycx1
hCD8 transmembrane/intracytoplasmic domains

FIG. 15(A)

```
-65  AGCTTCAGGA TCCTGAAAGG TTTTGCTCTA CTTCCTGAAG ACCTGAACAC
-15  CGCTCCCATA AAGCCATGGC TTGCCTTGGA TTTCAGCGGC ACAAGGCTCA
36   GCTGAACCTG GCTACCAGGA CCTGGCCCTG CACTCTCCTG TTTTTTCTTC
86   TCTTCATCCC TGTCTTCTGC AAAGCAATGC ACGTGGCCCA GCCTGCTGTG
136  GTACTGGCCA GCAGCCGAGG CATCGCCAGC TTTGTGTGTG AGTATGCATC
186  TCCAGGCAAA GCCACTGAGG TCCGGGTGAC AGTGCTTCGG CAGGCTGACA
236  GCCAGGTGAC TGAAGTCTGT GCGGCAACCT ACATGATGGG GAATGAGTTG
286  ACCTTCCTAG ATGATTCCAT CTGCACGGGC ACCTCCAGTG GAAATCAAGT
336  GAACCTCACT ATCCAAGGAC TGAGGGCCAT GGACACGGGA CTCTACATCT
386  GCAAGGTGGA GCTCATGTAC CCACCGCCAT ACTACCTGGG CATAGGCAAC
436  GGAACCCAGA TTTATGTAAT TGATCCAGAA CCGTGCCCAG ATTCTGACTT
486  CCTCCTCTGG ATCCTTGCAG CAGTTAGTTC GGGGTTGTTT TTTTATAGCT
536  TTCTCCTCAC AGCTGTTTCT TTGAGCAAAA TGCTAAAGAA AAGAAGCCCT
586  CTTACAACAG GGGTCTATGT GAAAATGCCC CCAACAGAGC CAGAATGTGA
636  AAAGCAATTT CAGCCTTATT TTATTCCCAT CAATTGAGAA TT
```

FIG. 15(B)

```
       -30        -20        -10          1         11         21
MACLGFQRHK AQLNLATRTW PCTLLFFLLF IPVFCKAMHV AQPAVVLASS RGIASFVCEY
                                       *

        31         41         51         61         71         81
ASPGKATEVR VTVLRQADSQ VTEVCAATYM MGNELTFLDD SICTGTSSGN QVNLTIQGLR

        91        101        111        121        131        141
AMDTGLYICK VELMYPPPYY LGIGNGTQIY VIDPEPCPDS DFLLWILAAV SSGLFFYSFL
                                             *

       151        161        171        181
LTAVSLSKML KKRSPLTTGV YVKMPPTEPE CEKQFQPYFI PIN
        *
```

FIG. 16

-36 AAGCTTCGAG CCAAGCAGCG TCCTGGGGAG CGCGTCATGG CCTTACCAGT
15 GACCGCCTTG CTCCTGCCGC TGGCCTTGCT GCTCCACGCC GCCAGGCCGA
65 GCCAGTTCCG GGTGTCGCCG CTGGATCGGA CCTGGAACCT GGGCGAGACA
115 GTGGAGCTGA AGTGCCAGGT GCTGCTGTCC AACCCGACGT CGGGCTGCTC
165 GTGGCTCTTC CAGCCGCGCG GCGCCGCCGC CAGTCCCACC TTCCTCCTAT
215 ACCTCTCCCA AAACAATCCC AAGGCGGCCA AGGGGCTGGA CACCCAGCGG
265 TTCTCGGGCA AGAGGTTGGG GGACACCTTC GTCCTCACCC TGAGCGACTT
315 CCGCCGAGAG AACGAGGGCT ACTATTTCTG CTCGGCCCTG AGCAACTCCA
365 TCATGTACTT CAGCCACTTC GTGCCGGTCT TCCTGCCAGC GAAGCCCACC
415 ACGACGCCAG CGCCGCGACC ACTAACACCG GCGCCCACCA TCGCGTCGCA
465 GCCCCTGTCC CTGCGCCCAG AGGCGTGCCG GCCAGCGGCG GGGGGCGCAG
515 TGCACACGAG GGGGCTGGAC TTCGCCTGTG ATATCTACAT CTGGGCGCCC
565 CTGGCCGGGA CTTGTGGGGT CCTTCTCCTG TCACTGGTTA TCACCCTTTA
615 CTGCAACCAC AGGAACCGAA GACGTGTTTG CAAATGTCCC CGGCCTGTGG
665 TCAAATCGGG AGACAAGCCC AGCCTTTCGG CGAGATACGT CTAACCCTGT
715 GCAACAGCCA CTACATGAAT TCC

FIG. 17

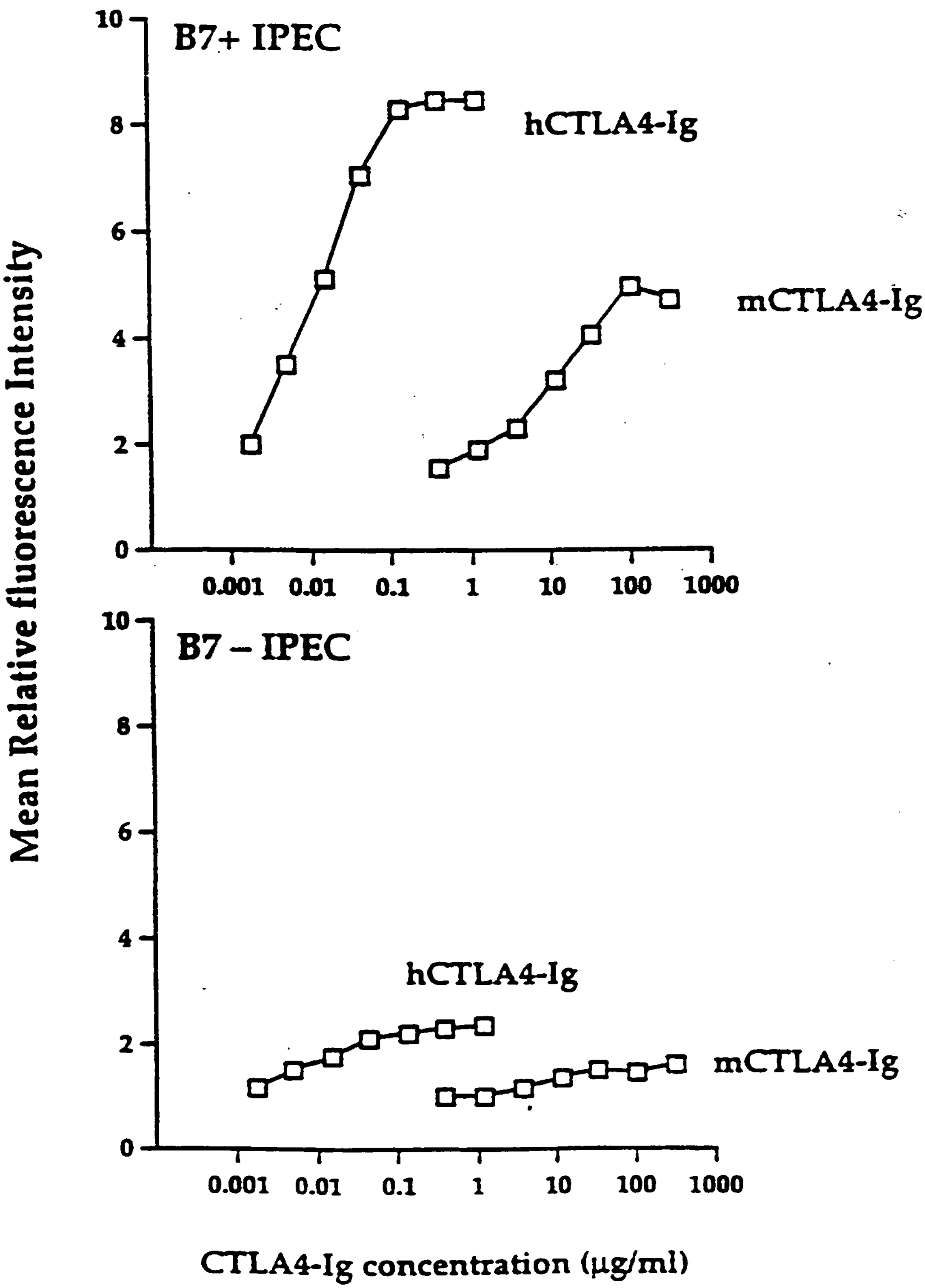

B7+ IPEC
hCTLA4-Ig
mCTLA4-Ig
B7 – IPEC
hCTLA4-Ig
mCTLA4-Ig
Mean Relative fluorescence Intensity
0
2
4
6
8
10
0.001 0.01 0.1 1 10 100 1000
CTLA4-Ig concentration (µg/ml)

FIG. 18

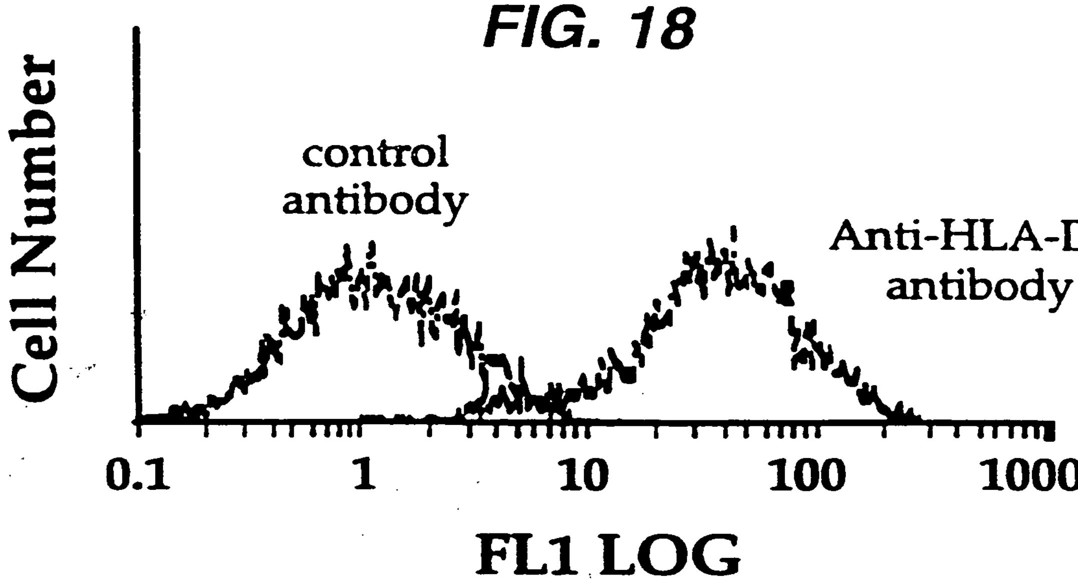

Cell Number
control antibody
Anti-HLA-DR antibody
0.1
1
10
100
1000
FL1 LOG

FIG. 19

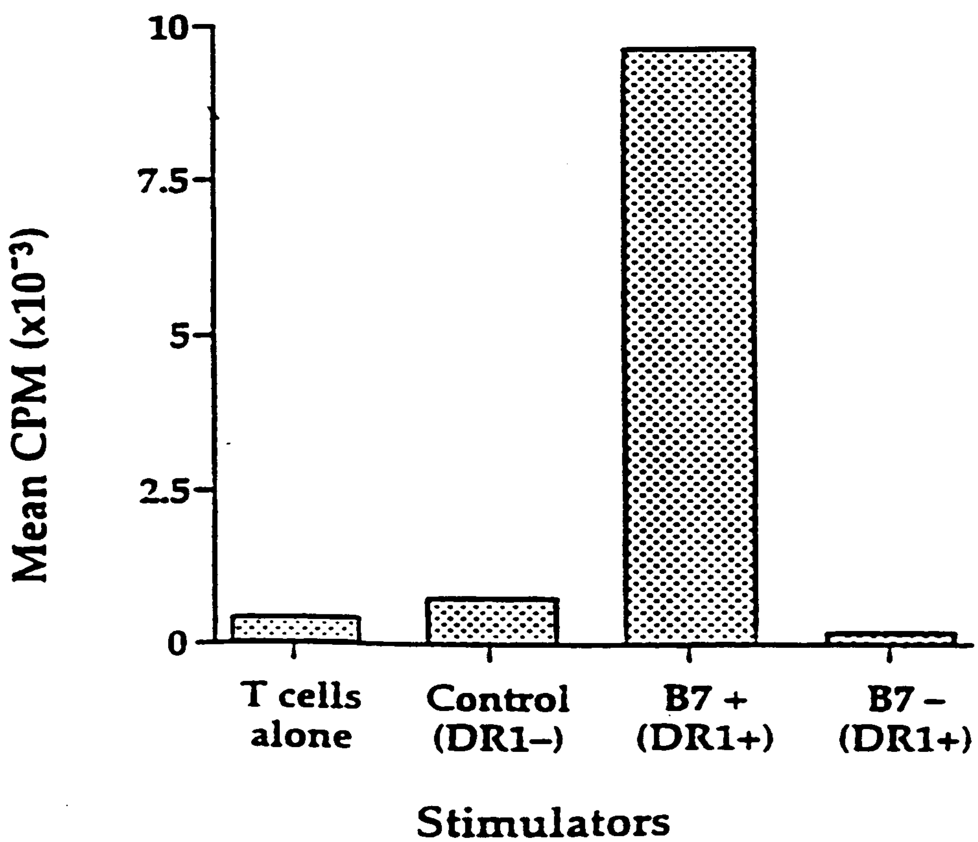

Mean CPM (x10^{-3})
10
7.5
5
2.5
0
T cells alone
Control (DR1−)
B7 + (DR1+)
B7 − (DR1+)
Stimulators

FIG. 20

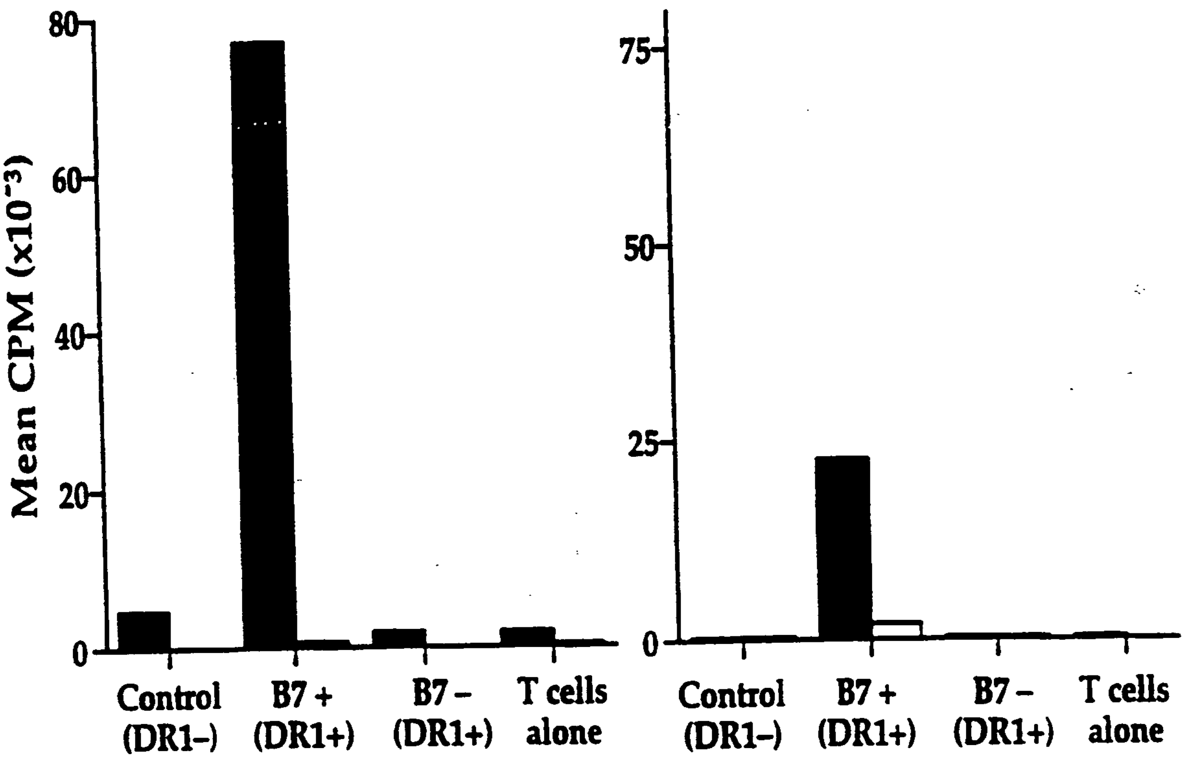
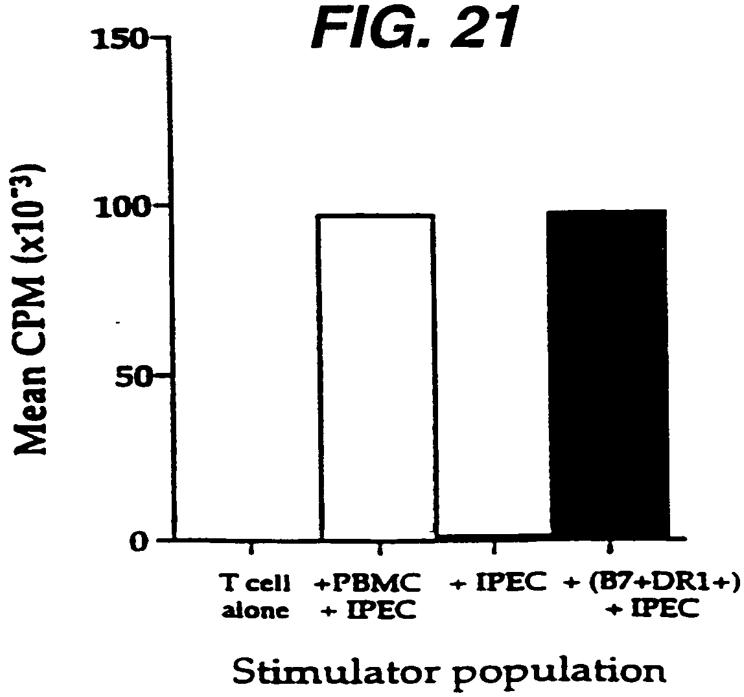

Mean CPM (x10⁻³)
80
60
40
20
0
Control (DR1-)
B7 + (DR1+)
B7 - (DR1+)
T cells alone
75
50
25
0
Control (DR1-)
B7 + (DR1+)
B7 - (DR1+)
T cells alone

FIG. 21


Mean CPM (x10⁻³)
150
100
50
0
T cell alone
+PBMC + IPEC
+ IPEC
+ (B7+DR1+) + IPEC
Stimulator population

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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C12N 15/12, 15/13, A61K 38/17, C12N 5/10, C07K 16/28, 14/705, A01K 67/027, A61K 31/70, 39/395, 35/14, 48/00

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9809280.2 30 April 1998 (30.04.98) GB

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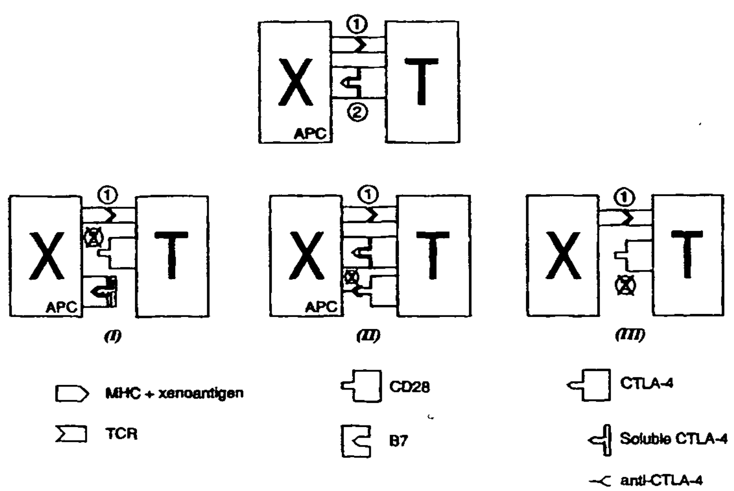
(54) Title: IMMUNOSUPPRESSION BY BLOCKING T CELL CO-STIMULATION SIGNAL 2 (B7/CD28 INTERACTION)



(57) Abstract

The invention provides means and methods for inhibiting T-cell mediated rejection of a xenotransplanted organ by blocking the delivery of co-stimulatory signal 2 (the B7/CD28 interaction) in order to prevent the activation of xenoreactive T-cells in the recipient. In a first aspect, co-stimulation is prevented by administration to the organ recipient of a soluble form of CTLA-4 from the xenogeneic donor organism. This preferentially binds B7 on the xenograft and blocks the interaction between B7 on the xenogeneic donor cells and CD28 on recipient T-cells. In a second aspect, co-stimulation is antagonised by expressing a ligand for CTLA-4 on the xenogeneic donor cells. This ligand binds to CTLA-4 on activated T-cells of the recipient and antagonises signal 2. In a third aspect, co-stimulation is prevented by expressing recipient organism MHC class II on the surface of the cells of the xenogeneic donor organ. The donor cells are thus able to present xenoantigens to a recipient T-cell in the context of self-MHC class II. If the donor cells do not express B7, or if B7 is blocked, the xenoreactive recipient T-cell becomes anergic.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/01350

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 2-4, 14 and 24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,2 (partially); 3-7 (completely)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-2 (partially), 3-7 (completely)

   Claims 1-2 partially concerning biological reagents and methods for inhibiting T-cell mediated rejection of xenotransplants in so far CTLA4 or CTLA4-fusion proteins are concerned; claims 3-7 completely in so far methods and reagents as above as well as proteins comprising the SEQ ID:1 and nucleic acids encoding it.

2. Claims: 1-2 (partially), 8-17 (completely)

   Claims 1-2 partially concerning biological reagents and methods for inhibiting T-cell mediated rejection of xenotransplants in so far CTLA4 binding proteins are concerned; claims 8-17 completely in so far methods and reagents as above as well as proteins and nucleic acids encoding CTLA4 binding proteins, cells and tissues expressing them and their uses in methods for rendering tissues suitable for xenotransplantation.

3. Claims: 1-2 (partially), 18-28 (completely)

   Claims 1-2 partially concerning cells and tissues which express MHC class II of different organisms capable to inhibit T-cell mediated rejection of xenotransplants; claims 18-28 completely in so far cells and tissues as above as well as animals comprising said cells or tissues and methods for rendering tissues suitable for xenotransplantation by expressing MHC class II on their surface.

INTERNATIONAL SEARCH REPORT

Interr. ıal Application No
PCT/GB 99/01350

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/13 A61K38/17 C12N5/10 C07K16/28 C07K14/705 A01K67/027 A61K31/70 A61K39/395 A61K35/14 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LENSCHOW D ET AL: "Long term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig" SCIENCE, vol. 257, 7 August 1992, pages 789-92, XP002021689 cited in the application see the whole document ---	1-7
X	COHEN J: "Mounting a targeted strike on unwanted immune responses" SCIENCE, vol. 257, 7 August 1992, page 751 XP002123149 cited in the application see middle column, line 3 - line 56 --- -/--	1-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 November 1999	22. 02. 2000

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/01350

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 34320 A (REGENTS OF THE UNIVERSITY OF MINNESOTA) 21 December 1995 see middle column, line 3; claims 17-20 -----	1-7

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/01350

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9534320 A	21-12-1995	AU 2701895 A	05-01-1996
		CA 2191733 A	21-12-1995
		EP 0784482 A	23-07-1997
		JP 10501815 T	17-02-1998

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